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Dissertação de Mestrado
Mestrado em Genética Molecular

Trabalho efetuado sob a orientação da
Professora Doutora Isabel Alexandra Duarte Ferreira Lopes Correia
e da
Professora Doutora Célia do Sacramento Santos Pais

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DECLARAÇÃO

Nome: Tânia Isabel Pimenta Lima
Endereço eletrónico: taniaisabellima@gmail.com
Bilhete de Identidade.: 14174670

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Orientadores
Professora Doutora Isabel Alexandra Duarte Ferreira Lopes Correia
Professora Doutora Célia do Sacramento Santos Pais

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Abstract

Yeasts of the genus *Candida* are important human pathogens. A new species, *Candida bracarensis* was recently described revealing phenotypic and genetic similarities with *Candida glabrata*, a widespread pathogen, and also with *Saccharomyces cerevisiae*. The incidence of *C. bracarensis* has increased globally being now regarded as an emergent fungal pathogen. However, little is known about its characteristics as a pathogenic agent and the interaction with its host. The aim of this work was to compare *C. bracarensis* with *C. glabrata* and *S. cerevisiae* concerning their interaction with the host. We observed that *C. glabrata* was phagocytosed at higher rates than *C. bracarensis* by murine and human macrophages, but both species similarly stimulated the production of reactive oxygen species (ROS). *S. cerevisiae* induced the lowest phagocytosis rate and production of ROS. Other differences observed were a higher content of (1,3)-linked glucose, which is related with the content in β-1,3-glucans, in *C. bracarensis* cell wall. In accordance, *C. bracarensis* yeasts stained with an anti-β-1,3-glucan monoclonal antibody showed a significantly brighter signal than *C. glabrata* and *S. cerevisiae*, as assessed by flow cytometry and confocal microscopy. Moreover, *C. bracarensis* showed the highest percentage of relative cell wall porosity, as evaluated by using DEAE-dextran/poly-L-lysine polycations. Additionally, increased levels of the proinflammatory cytokines IL-12, TNF-α, IL-1β, and IL-23 were generally detected in the culture supernatants of murine bone marrow-derived dendritic cells (BMDCs) and macrophages (BMMs) stimulated with *C. bracarensis* as compared to cultures incubated with the other two yeast species. The recognition of the former yeast species by Dectin-1 would thus be in agreement with the observed increased IL-23 and also TNF-α production. Indeed, anti-Dectin-1 blockage significantly decreased the levels of TNF-α in both BMDCs and BMMs stimulated with *C. bracarensis*, but not with *C. glabrata* and *S. cerevisiae*, implicating this pattern recognition receptor in *C. bracarensis* regonition and signaling. Considering all the above results we hypothesize that *C. bracarensis* cell wall higher porosity might account for higher accessibility to β-1,3-glucans thereby facilitating yeast recognition by the C-type lectin Dectin-1, resulting in the induction of a strong response by the host immune system that could contribute to a faster elimination of this pathogen. Although these results do not by themselves fully elucidate the disparate incidence of *C. glabrata vs C. bracarensis*, they nevertheless contribute to better understand the differences on the interaction of these two related species with the host.

Resumo

As leveduras do género *Candida* são importantes agentes patogénicos em infeções fúngicas humanas. Recentemente foi descrita uma nova espécie, *Candida bracarensis,* que se assemelha tanto fenotípica como genotipicamente com *Candida glabrata* e também com *Saccharomyces cerevisiae*. A incidência desta nova espécie tem vindo a aumentar, estando entre os fungos considerados emergentes. Contudo, muito pouco se conhece acerca das suas características como patogéneo e sobre a sua interação com o hospedeiro. Assim, o objetivo deste projeto foi comparar *C. bracarensis*, *C. glabrata* e *S. cerevisiae* no que diz respeito aos mecanismos de interação com o hospedeiro. *C. glabrata* foi mais fagocitada do que *C. bracarensis* por macrófagos humanos e de ratinho. No entanto, ambas as espécies estimularam semelhante produção de espécies reactivas de oxigénio (ROS). *S. cerevisiae* foi a espécie menos fagocitada e a que estimulou menor produção de ROS. Foi ainda observado que a parede celular de *C. bracarensis* era constituída por um elevado teor de β-1,3-glucanas, o que está de acordo com o sinal significativamente mais intenso das células de *C. bracarensis*, em comparação com as de *C. glabrata* e *S. cerevisiae*, quando marcadas com um anticorpo anti-β-1,3-glucanas e analisadas por citometria de fluxo e por microscopia confocal. *C. bracarensis* foi também a espécie que apresentou a maior percentagem relativa de porosidade da parede celular, determinada por um ensaio com os poli-catiões DEAE-dextrano e poli-L-lisina. Adicionalmente, os níveis de citocinas pro-inflamatórias IL-12, TNF-α, IL-1β, e IL-23 nos sobrenadantes de culturas de células dendríticas e de macrófagos de ratinho derivados da medula óssea foram mais elevados quando infetados com *C. bracarensis* do que com as outras duas espécies de levedura. O reconhecimento de *C. bracarensis* pelo receptor Dectin-1 poderá explicar a elevada produção de IL-23 e TNF-α induzida por esta espécie. De facto, o bloqueio de Dectin-1 levou a uma marcada redução dos níveis de TNF-α em resposta a *C. bracarensis,* salientando o impacto deste receptor no reconhecimento desta espécie e na posterior sinalização. Coloca-se assim como hipótese que a elevada porosidade da parede celular de *C. bracarensis* possa permitir uma maior acessibilidade aos β-1,3-glucanas e facilitar o seu reconhecimento pela lectina Dectin-1, o que poderia contribuir para uma eliminação mais rápida e eficiente deste agente patogénico. Ainda que estes resultados não expliquem por si só a disparidade encontrada na incidência de *C. glabrata* e *C. bracarensis*, contribuem no entanto para um melhor conhecimento da interação destas duas espécies com o hospedeiro.

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ANOVA	Analysis of variance;
APC	Antigen presenting cell
ATCC	American Type Culture Collection
BMDC	Bone marrow derived dendritic cells
BMM	Bone marrow derived macrophages
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CFU	Colony forming unit
CFW	Calcofluor White
CLR	C-type lectin receptor
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule 3 grabbing non-integrin
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenadiaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay;
FACS	Fluorescence activated cell sorting; flow cytometry
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI-	Glycosylphosphatidylinositol-anchored
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

kDa	Kilodalton
LCCM	L929 cell conditioned medium
LPS	Lipopolysaccharide
mAb	Monoclonal antibody;
MDM	Monocytes-derived macrophages
MFI	Mean Fluorescence intensity
MHC	Major histocompatibility complex
MR	Mannose receptor
NF-kB	NF-kB Nuclear factor kappa-light-chain-enhancer of activated B cells
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
ROS	Reactive oxygen species
RPM	Murine residente peritoneal macrophages
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
Sap	Secreted aspartyl protease
SD	Standard deviation
TB	Trypan blue
Th	T helpher
TLR	Toll like receptor
TNF-α	Tumor necrosis factor- alpha
UK	United Kingdom
USA	United States of America
YPD	Yeast extract, Peptone, Dextrose

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Chapter 1

General Introduction

1. Fungal infections: characterization and clinical importance

Fungi represent a large, diverse and widespread group of heterotrophic eukaryotes. These organisms are very proficient at sensing their surrounding and responding to signals that promote their survival in changing environments. Furthermore, they can interact with plants, animals or humans in multiple ways, establishing symbiotic, commensal, latent or pathogenic relationships (Madigan *et al.,* 2011). As a result, in the last decades, human fungal infections incidence has risen especially in immunocompromised and hospitalized individuals. The morbidity and mortality associated with these infections are substantial, and it is clear that fungal diseases have emerged as important public health problems. McNeil *et al.*, (2001) analyzed the trends in infectious disease mortality in the United States and found a dramatic increase in multiple-cause mortality due to mycoses, from 1557 deaths in 1980 to 6534 deaths in 1997. The majority of the mycose-related deaths are associated with *Candida, Aspergillus,* and *Cryptococcus* infection (Krcmery and Barnes, 2002; Pfaller and Diekema, 2007; Silva *et al.,* 2012). There are several possible reasons for the elevated incidence of systemic fungal infections: i) an important one might be the increase in lifespan in the populations of the developed world and the age related loss of immune-competence; ii) an increase of systemic fungal infections was probably also due to more intensive treatment schemes for haematological and oncological patients causing prolonged neutropenic phases; iii) finally, more effective antibacterial treatments allow patients with infections to survive longer without necessarily overcoming the underlying diseases and thus leaving them susceptible to other opportunistic infections (Pfaller and Diekema, 2007; Sardi *et al.,* 2013).

Of the fungi regarded as human pathogens, the members of the genus *Candida* are the most frequently recovered from human fungal infections. The genus *Candida* contains over 150 heterogeneous species, but only a minority has been implicated in human candidiasis. Of the *Candida* species isolated from humans, *Candida albicans* is the most prevalent under both healthy and disease conditions. However, *Candida glabrata, Candida tropicalis, Candida krusei* and *Candida parapsilosis* are involved in pathogenic episodes too, and the number of infections due to non-*albicans Candida* species has increased significantly (Krcmery and Barnes, 2002; Mayer *et al.,* 2013).

2. *Candida* infections: overview

Candida species belong to the normal microbiota of an individual's mucosal oral cavity, gastrointestinal and genitourinary tracts but, under certain circunstances, can be responsible for various clinical manifestations from mucocutaneous overgrowth to bloodstream infections (Sampaio *et al.* 2010; Gow and Hube, 2012; Wang *et al.,* 2014). Transition from colonization to mucosal and/or systemic infection is partly explained by modern medical progress, relying on large-spectrum antibiotics, immunosuppressive chemotherapy, and devices such as catheters, all of which have been shown to predispose to invasive candidiasis (Gabaldón *et al.,* 2013). In order to cause infection, the opportunistic pathogens need to invade different host sites, evade the immune system, and survive and reproduce within the host. Furthermore, in systemic infections, they spread to new tissues and organs (Yang, 2003; Haynes, 2001; Silva *et al.,* 2012).

2.1 *Candida glabrata* and its related species *Candida bracarensis*

In the last decades, the number of infections due to *C. glabrata* has increased considerably (Rodrigues *et al.*, 2014). Historically, *C. glabrata* has always been referred to as a non-pathogenic yeast from the normal saprophytic flora of healthy subjects and rarely were described severe infections caused by this species in humans (Fidel *et al.*, 1999; Brieland *et al.,* 2001; Kaur *et al.*, 2005). However, due to the widespread and increasing use of immunosuppressive agents, in conjunction with the implementation of broad-spectrum antimycotic therapy, the frequency of mucosal and systemic infections caused by *C. glabrata* increased significantly, making it the second or third most common cause of candidiasis (Kullberg and Arendrup, 2015). *C. glabrata* is able to colonize commensally the mouth, esophagus, intestines, and vagina mucosal surfaces, but still little is known about its interaction with the host and which are the protective host defence mechanisms. It is expected that host mechanisms control *C. glabrata*, by suppressing the expression of its pathogenic properties, thus preventing infection (Kramer *et al.,* 2006; Roetzer *et al.,* 2011). *C. glabrata* is haploid and presents a yeast form. Until now, episodes of hyphae formation in *C. glabrata* have not been described and, hardly ever, formation of pseudohyphae was observed (Csank and Haynes 2000; Jandric and Schuller 2011; Brunke and Hube 2013). Compared to other *Candida* species, *C. glabrata* appears to be one of the most vigorous, being able to survive on inanimate surfaces for more than 5 months, while the viability of *C. albicans* is limited to 4 months and *C. parapsilosis* cells die after 2 weeks in similar conditions (Kramer *et al.,*

2006; Roetzer *et al.,* 2011). The genome of *C. glabrata* type strain (CBS138^T/American Type Culture Collection, ATCC 2001^T), was sequenced and despite its name, this yeast is phylogenetically closer to the model yeast *Saccharomyces cerevisiae* than to *C. albicans*, and is part of the Saccharomycetaceae family (Figure 1) (Fidel *et al.,* 1999; Dujon *et al.,* 2004; Gabaldón *et al.,* 2013). *C. glabrata* shares a common ancestral with several *Saccharomyces* species, but has lost many more genes during evolution than *S. cerevisiae* from their common ancestor. These changes in *C. glabrata* genome decreased traces of whole genome duplication (WGD) to a minimum and led to the complete loss of some metabolic pathways (Dujon *et al.,* 2004). Nevertheless, expansion of other genes gave *C. glabrata* other evolutionary advantages (Roetzer *et al.*, 2011). Namely, expansion of genes involved in cell wall organization occurred in *C. glabrata*, possibly facilitating adherence to a broad spectrum of surfaces. It is important to emphasize that the gain and loss of these relevant genes might be crucial for functional differentiation between species and could be related to *C. glabrata* 's adaptation as a mammalian commensal (Butler *et al.,* 2004; Hittinger *et al.,* 2004; Roetzer *et al.,* 2011). Thus, robust adherence, resistance to antifungal drugs and to stress agents, and enhanced ability to sustain prolonged starvation render *C. glabrata* superior to *S. cerevisiae* in causing disease. *C. glabrata* is also able to survive inside phagocytic cells for large periods of time which might support the establishment of disseminated infection and thus directly relate to its success not only as a commensal but also as a pathogen (Roetzer *et al.,* 2011; Rodrigues *et al.,* 2014).

During a study of candidiasis in Portugal, it was discovered that one of the clinical isolates initially identified as *C. glabrata*, and phenotypically similar to this species, showed some different genotypic characteristics. After sequencing of the ITS regions and the D1/D2 region of ribosomal DNA genes, it was found that the degree of divergence was considerably high, which led to the description of a new species which was named *Candida bracarensis.* The type strain, 153 M^T (CBS 10154^T), was isolated from a case of vaginal candidiasis in a medical institution of Braga, Portugal (Correia *et al.,* 2006). The number of *C. bracarensis* isolates described is still low. Following the publication of the first two isolates as a cause of vulvovaginal candidiasis (VVC) in Portugal and from a blood culture in the United Kingdom in 2006 (Correia *et al.,* 2006), only fourteen more have been reported. One isolate was identified in The John Hopkins Clinical Mycology Laboratory (Maryland) collection (Page *et al.,* 2006), three were isolated from the throat and stool of two oncology patients and from a pelvic abscess in Baltimore (Bishop *et al.*, 2008), two isolates have been recovered from a study of *C. glabrata* bloodstream isolates in San Francisco (Lockhart *et al.,*

2009), and two bloodstream strains were isolated from a bone marrow transplant patient, all from the United States (Warren *et al.*, 2010). Three more isolates were found in a Spanish collection of yeasts (Cuenca-Estrella *et al.*, 2011) and one isolate was collected in a VVC patient in China (Li *et al.*, 2014). Besides these published isolates, one *C. bracarensis* isolate was identified by Center Hospitalier J. MIJOZ - Laboratoire de Bacteriologie, and another by our group among *Candida sp.* isolates from Instituto Nacional de Saúde Ricardo Jorge, Lisbon, Portugal.

C. bracarensis belongs to the *glabrata* group (Figure 1) which, apart from *C. glabrata*, includes two other species usually isolated from human infections (*C. bracarensis* and *C. nivariensis*) and one from the environment (*Nakaseomyces delphensis*, formerly *Kluyveromyces delphensis*) (Gabaldón *et al.,* 2013). Although some studies have shown that the emerging pathogen *C. bracarensis* is more closely related to the non-pathogenic *N. delphensis* than to the most frequently isolated pathogen *C. glabrata*, (Correia *et al.*, 2006; Gabaldón *et al.,* 2013), the similarities shared by *C. glabrata* and *C. bracarensis* may represent adaptations that may have facilitated, even if not directly triggered, the emergence of pathogenicity towards humans (Gabaldón *et al.*, 2013. In all infections attributed to *C. bracarensis* every isolate was initially identified as *C. glabrata* based on the routinely used phenotypic tests (Correia *et al.*, 2006; Bishop *et al.,* 2008; Lockhart *et al.,* 2009; Warren *et al.,* 2010; Cuenca-Estrella *et al,.* 2011; Li *et al,.* 2014). This highlights the high grade of phenotypic similarities shared by *C. bracarensis* and *C. glabrata* and emphasizes the problem of misidentification between these closely related *Candida* species (Romeo *et al.,* 2009). Thus, the true incidence of this organism in cases of candidiasis is yet to be determined (Warren *et al.,* 2010).

Similar to other *Candida* species, *C. bracarensis* has been recovered from multiple body sites, especially from mucosal surfaces, and is clearly associated with colonization and infection (Correia *et al.,* 2006; Bishop *et al.,* 2008; Lockhart *et al.,* 2009). So far, little is known about this species regarding its prevalence, susceptibility patterns, and clinical significance. However, physiological and biochemical tests demonstrate a great similarity between *C. glabrata* and *C. bracarensis.* Indeed, *C. bracarensis* and *C. glabrata* are morphologically indistinguishable except for the color change on CHROMagar and lysine assimilation (Warren *et al.,* 2010). *C. bracarensis* can be presumptively identified based on the production of white colonies on CHROMagar agar, microscopic features like small budding yeast cells without hyphae or pseudohyphae, and a rapid trehalose test. Identification can be further confirmed by DNA sequence analysis targeting the ITS and/or D1/D2 regions (Correia *et al.,* 2006; Warren *et al.,* 2010; Esposto *et al.*, 2013).

Relatively to antifungal resistance, Warren and co-workers (2010) have shown that two of the eight *C. bracarensis* isolates tested for antifungal drug susceptibility were drug resistant, being one resistant to amphotericin B and another resistant to all the azoles. It was also reported that *C. bracarensis* were able to form biofilms composed by proteins and carbohydrates, had hemolytic activity, one isolate had extracellular proteinase activity, and were unable to produce phospholipase and filamentous forms (Moreira *et al.,* 2015).

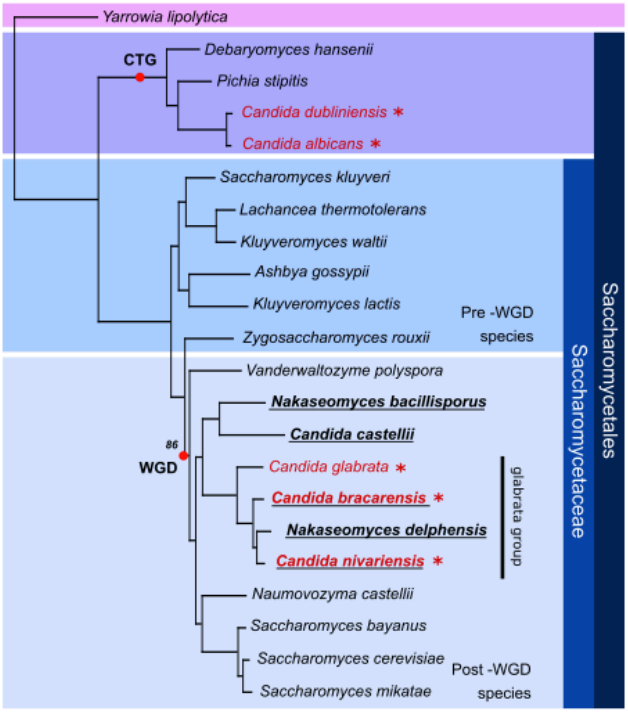

Figure 1: Phylogenetic tree of 22 *Saccharomycotina* species. The tree was constructed from maximum likelihood analysis based on concatenated alignment of one-to-one orthologs of 603 widespread genes. Species names in red and with an asterisk indicate human fungal pathogens. Underlined species names correspond to the newly sequenced species. Important evolutionary events such as the Whole Genome Duplication (WGD) or the genetic code transition in the *Candida* clade (CTG) are marked on the tree (Gabaldón *et al.,* 2013).

3. *Candida* virulence factors

Candida pathogenicity is a complex and higly regulated multifactorial process (Brown *et al.*, 2007). Several studies suggest that *C. glabrata* has few virulence tributes taking into account its relatively less exuberant pathogenic nature (De Bernardis *et al.*, 1996; Fidel *et al.,* 1996). However, the high mortality rate due to *C. glabrata* infections and the rapidity of the spread of disease would argue to the contrary (Fidel *et al.*, 1999). *C. glabrata* pathogenicity depends not only on its characteristics but also on the host physiology conditions, specifically, on the state of the host immune system

(Rodrigues *et al.,* 2014). *C. glabrata* shares some virulence factors with *C. albicans* including phenotypic switching, biofilm formation and the ability to adhere to host tissues. Despite the lack of several virulence mechanisms characteristic of *C. albicans*, such as hypha formation and extracellular proteolytic activity, *C. glabrata* is capable of establishing successful disseminated infections (Rai *et al.,* 2012). If *C. glabrata* virulence is low, the lack of hyphae formation may be a contributing factor. Indeed, hypha formation is a recognized attribute of increased adherence and tissue invasion of *C. albicans,* as well as a means of increasing proteolytic enzyme elaboration and antigen modulation (Brunke and Hube, 2013). Secreted aspartyl proteinase production by *C. albicans* has been frequently associated with pathogenicity (Naglik *al.,* 2003), however, the contribute of these hydrolytic enzymes to *C. albicans* pathogenicity seems to highly depend on the type of infection (Lermann and Morschhäuser, 2008; Correia *et al*., 2010). In general, little is know about proteinase production by *C. glabrata*, but in the few studies available, *C. glabrata* was described as not presenting extracellular proteinase activity (Kaur *et al.,* 2005). Nevertheless, in a recent study *C. glabrata* was demonstrated to up-regulate members of the *C. glabrata* YPS gene family encoding putative GPI-linked aspartyl proteases upon contact with macrophages (Kaur *et al.,* 2007). The main role of Yps proteases is to remodel the cell surface by removal of certain GPI-CWPs in response to different host environments (Kaur *et al.,* 2007; Bairwa *et al.,* 2014; Supúlveda-González *et al.,* 2015).

Another virulence factor of *C. albicans* is phenotypic instability, termed "phenotypic switching", which allows isolates to switch colony phenotype without affecting the identifiable genotype (Yang, 2003). This feature, that contributes to the morphological and phenotypic plasticity of *C. albicans*, is implicated in the control of the sexual cycle and in virulence. Regulation of other virulence-associated traits (e.g. hyphal formation and secretion of proteinases) is affected by this virulence attribute (Kaur *et al.,* 2005). Phenotypic switching was also reported in *C. glabrata* (Csank and Haynes, 2000; Lachke *et al*., 2002). Intriguingly, in some patients infected with a single *C. glabrata* strain, dramatic differences were seen in the proportions of switch phenotypes at oral and vaginal locations, which suggest the potential selection of particular switch phenotypes at different anatomical sites (Brockert *et al.,* 2003). The ability to switch would allow rapid adaptation of the organism to different stressful environments, and is a proposed route for the generation of phenotypic diversity species (Kaur *et al.,* 2005).

Adherence is considered essential in the establishment of disease and contributes to the persistence of the organism within the host. Adherence of *Candida* species to host epithelial tissue

is thought to play an important role in the virulence of these organisms. Also, pathogenic yeasts exploit their ability to adhere to abiotic surfaces, such as plastic prostheses and catheters, to gain access to the bloodstream and internal organs of the patients (Kojic and Darouiche, 2004). In *C. albicans* adherence is mediated by numerous defined adhesins including those encoded by *HWP1*, expressed only on hyphae, and by members of the *ALS* (agglutinin-like sequence) gene family (Sundstrom, 1999; Sheppard *et al.*, 2004). The Als3 protein, in particular, seems to play a vital role in adhesion, since its deletion strongly reduces adherence to epithelial cells (Brunke and Hube, 2013). In *C. glabrata*, adherence is mediated largely by the *EPA* family of genes, which, like *HWP1* or *ALS* genes encode GPI-anchored cell wall proteins (Cormack et al., 1999). These adhesins of *C. glabrata* are related to the Flo proteins of *S. cerevisiae*, which are responsible for flocculation during the brewing process (Brunke and Hube, 2013). *C. glabrata* possesses a family of at least 23 *EPA* genes, which encode cell surface proteins capable of mediating adherence to epithelial cells and macrophages (Mundy and Cormack, 2009; Halliwell *et al.*, 2012; Brunke and Hube, 2013). Gabaldón and co-workers (2013) identified 12 chromosomes and 12 members of the *EPA* family in *C. bracarensis*. It has been postulated that the *EPA* genes could be differentially regulated in order to maximize adherence to different host cell types during infection (Halliwell *et al.*, 2012).

4. *Candida* cell wall

Different virulence factors are involved in *Candida* pathogenicity, but the role of the cell wall in the pathogenesis of the fungus cannot be undervalued. The cell wall is the dynamic and complex structure that first comes into contact with host cells and carries basic antigenic determinants of the fungus. Equally important is the contribution of the cell wall for the adherence of the pathogen and establishment of a connection with the host, which depends on the chemical composition and linkages of the cell wall polysaccharides (Ruiz-Herrera *et al.,* 2006; Hall and Gow, 2013; Hall, 2015).

The *Candida* cell wall is a coherent structure, made by ordered arrangement of its different components that provides the fungus protection against physical, chemical and biological aggression, and is responsible for its morphology (Ruiz-Herrera *et al.*, 2006; Hall and Gow, 2013; Alvarez, 2014). The general *Candida* cell wall is composed by 90% carbohydrate and 10% protein. As a generalization, carbohydrates dominate the immune recognition and proteins have a key role

in adhesive interactions with host cell surfaces (Gow and Hube, 2012). The three major forms of polysaccharides in *Candida* species are mannans, glycoprotein containing mannose in a variety of α and β linkage arrangements; β-glucans, which comprise mainly β-1,3-glucan and β-1,6-glucan; and chitin (poly-β-(1,4)-N-acetylglucosamine-GlcNAc) or its deacetylated form, chitosan (Galán-Díez *et al.,* 2010; Gow and Hube 2012). The cell wall inner layer is comprised of chitin and glucans that act as the cell's skeleton, and both of these molecules convey strength and shape to the cell wall. By contrast, the mannans and proteins of the outer cell wall layer are less structured, but have low permeability and porosity that are essential for cell adhesion, biofilm formation and ultimately to *Candida* virulence (Hall, 2015). These proteins are decorated with simple linear *O*-linked glycans and complex branched *N*-linked mannans. As these mannans are located on the periphery of the cell wall, they play key roles in innate immune recognition (Hall, 2015). Therefore, the mannan layer affects the resistance of the wall to attack by molecules of the host defence and the permeability of the wall to antifungal drugs, but does not influence cell shape (Gow and Hube, 2012). A representative scheme of *C. albicans* cell wall is shown in Figure 2. Global transcriptional and proteomic studies investigating the effects of environmental parameters identified that many of the cell wall proteins are differentially expressed in different environments (Bruno *et al.,* 2010). Growth in different environments also affects the carbohydrate content of the cell wall (Ene *et al.,* 2012). The presence or absence of specific proteins within the cell wall and the exposure of specific carbohydrates will affect cell wall structure and composition, ultimately affecting innate immune recognition (Ene *et al.,* 2012; Hall, 2015).

At the moment, no evidences of the presence of α-glucans in the *Candida* genus have been reported. (Ruiz-Herrera *et al.,* 2006) *Candida* cell wall only comprises β-glucans, and these are the most abundant polysaccharides of the fungal cell wall in general. They are polymers of glucose joined by β-1,3-glycosidic or β-1,6-glycosidic linkages. *Candida* cell wall contains both β-1,3 and β-1,6-glucans, but no mixed intrachain β-1,3/1,6 linkages (Ruiz-Herrera *et al.* 2006; Hall, 2015). In yeats the cell wall proteins are bound to the β-glucan/chitin inner layer through the lateral chains of β-1,6-glucan or, to a lesser extent, to β-1,3- glucan (Ruiz-Herrera *et al.,* 2006).

β-glucans have been reported to modulate inflammatory responses *in vitro* and *in vivo* (Nakagawa *et al.* 2003). The immunomodulatory effects of β-glucans are influenced by their degree of branching and their tertiary structure, and different types of glucans have different biological effects (Bain *et al.,* 2014; Bonfim-Mendonça *et al.* 2014).

The synthesis of β-glucans is a complex reaction involving several enzymes located at different cell compartments. Because of that, glucan synthases constitute the target of important inhibitors of glucan synthesis, and therefore of wall growth (Ruiz-Herrera *et al.,* 2006). To inhibit fungal growth, various efficacious antibiotics have been developed to interfere with cell wall synthesis by targeting β-1,3-glucan synthase (Selvakumar *et al.,* 2006). However, no antifungal antibody that could inhibit β-1,3-glucan synthase activity has ever been reported. Glucose synthase is composed by two related catalytic subunits, Fks1p and Fks2p, and a regulatory sub-unit, Rho1p small GTPase (Inoue *et al.,* 1995; Drognova *et al.,* 1996; Qadota *et al.,* 1996). All components of the glucose synthase are localized on the plasma membrane at the site of cell wall remodelling (Utsugi *et al.,* 2002).

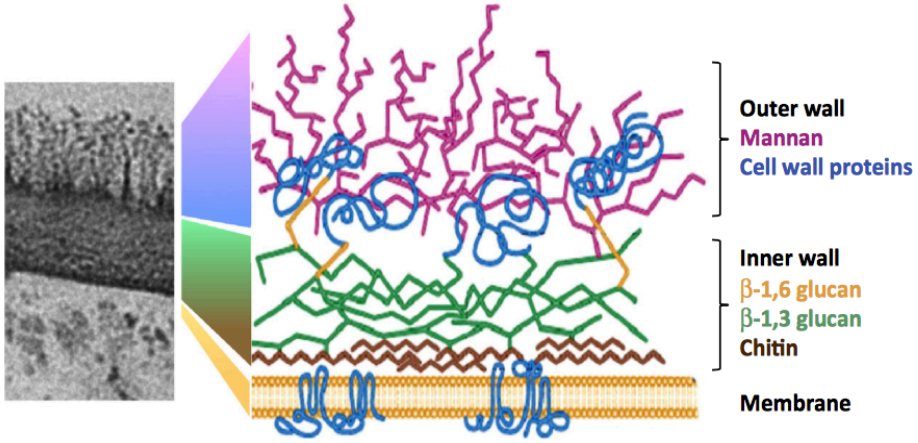

Figure 2: The structure of the *Candida albicans* cell wall (adapted from Brown *et al.,* 2014). The three major forms of polysaccharides in *Candida* species are: (a) mannans, (b) β-glucans, which comprise mainly β-1,3-glucan and β-1,6-glucan, and (c) chitin .

Chitin is a linear polysaccharide made of several units of N-acetylglucosamine. Due to this crystalline arrangement, chitin is one of the most insoluble natural products, and this explains why its linkages to β-1,3-glucan form the basic cell wall scaffold to which mannoproteins are covalently associated (Ruiz-Herrera *et al.* 2006, Levitz, 2010). Although chitin only comprises a small amount of the fungal cell wall (3% dry weight), subtle changes in its concentration can have a large impact on fungal biology. An increase in chitin content from 3% to 10% greatly impacts on antifungal sensitivity and recognition by the innate immune system (Mora-Montes *et al,.* 2011).

Treatment of *Candida* with caspofungin, an echinocandin that inhibit the synthesis of β-(1,3)-glucan, has been reported to cause a compensatory increase in chitin content (Walker *et al.,* 2013). This activation of chitin synthesis was observed in isolates of *C. albicans*, in response to

caspofungin treatment, however, *C. glabrata* isolates demonstrated no exposure-induced changes in chitin content (Walker *et al.,* 2013). Remarkably, the chitin response is fully reversible upon removal of cell wall stress, complicating the evaluation of the precise significance of this phenomenon *in vivo*.

5. Interaction between *Candida spp*. and host immune cells

The host immune response to fungal infections comprises diverse mechanisms and involves both innate and adaptive immunity. At the same time that the immune system is eliminating pathogens and toxic substances, it must avoid responses that produce excessive damage of self-tissues or even that might eliminate beneficial commensal microorganisms (Chaplin *et al.,* 2010). The immune system uses a complex array of protective mechanisms that rely on detecting structural features of the pathogens that mark them as distinct from host cells, to control and usually eliminate these organisms and toxins. Such host-pathogen discrimination is essential to permit the host to eliminate the threat without damaging its own tissues (Romani, 2011).

5.1 Overall of Immune System: organization and role

The immune system is a collection of barriers, cells and soluble factors that interact and communicate with each other in an extraordinary complex way (Murphy et al., 2007). Any discussion about the immune response usually begins with the physiologic and anatomic barriers that prevent pathogens from entering the body, destroy them after they enter, or flush them out before they can establish themselves. They are part of the body's most basic defence mechanisms and are associated with constitutive mechanisms of innate defence (Sajjad *et al.* 2010; Romani, 2011). These barriers include skin, mucosal epithelial cells of respiratory, gastrointestinal and genitourinary tracts, and are in constant interaction with pathogens (Romani, 2011). The anatomic barrier defences provide the crucial primary line of defence against pathogens and are continuously working to protect against a broad range of pathogens, although they are not, by themselves, a response to infections (Turvey and Broide, 2010). Whenever that first line of defence is breached, the host innate immune system plays a crucial role in the early recognition of invading pathogens which will subsequently trigger a proinflammatory response that will attack the invading substances (Traynor and Huffnagle, 2001).

Despite a certain lack of specificity, innate immunity effectively distinguishes self from non-self and activates adaptive immune mechanisms by the provision of specific signals (Medzhitov and Janeway, 1997; Romani, 2004). The innate immune system confers rapid recognition of microbial infections through a limited repertoire of germ line-encoded receptors that recognize a group of conserved molecular patterns (pathogen-associated molecular patterns, PAMPS) common to broad groups of microbial agents (Janeway and Medzhitov, 2002; Roeder *et al.,* 2004; Romani, 2004; Netea, *et al.* 2015). The innate immune response is mediated mainly by phagocytic cells such as granulocytes, macrophages, and dendritic cells (DC) (Aderem, 2003; Mogensen, 2009). However, other factors such as cytokines and chemokines, the complement system, and also natural killer cells, play an important role in innate immunity (Mogensen, 2009). Some participants of the innate immunity, such as dendritic cells and the complement system, may assist the induction of adaptive immunity (Dunkelberger and Song, 2010; Steinman and Hemmi, 2006). The adaptive immune system is responsible for the elimination of pathogens in a later phase of infection and is involved in the generation of immunological memory, a unique feature of the adaptive immune system (Murphy *et al.* 2007). The hallmarks of adaptive immunity are its ability to learn, adapt and remember (Alberts *et al.* 2002).

There are two broad classes of adaptive responses, antibody responses and cell-mediated immune responses, which are carried out by B and T lymphocytes, respectively. In antibody responses, B cells are activated to secrete antibodies. The antibodies circulate in the bloodstream and permeate the other body fluids, where they bind specifically to the antigen that stimulated their production. Binding of antibodies to their specific antigens may inactivate pathogens by blocking their ability to bind to receptors on host cells. Antibody binding also marks invading pathogens for destruction, mainly by making it easier for phagocytic cells of the innate immune system to ingest them (Alberts *et al.* 2002; Bonilla and Oettgen, 2010). Summing up, to protect the individual effectively against disease, the immune system must fulfil four main tasks. The first is the immunological recognition, where the presence of invading pathogens and establishment of infection must be detected. The second task is the control of the infection and, if possible, the complete elimination of the pathogen which brings into play immune effector functions. Immune regulation, or the ability of the immune system to self-regulate, is thus an important feature of immune responses and would constitute the third task. The fourth task is to protect the individual against recurrent disease due to the same pathogen and is related to the immunological memory.

5.2 Pathogen recognition strategies and innate sensing

The immune system does not remain ignorant of commensal or ubiquitous fungi, and thus, a fine balance between pro and anti-inflammatory signals is required to maintain a stable host–fungus relationship, the disruption of which can have pathological consequences (Romani, 2011).

The first step in triggering an immune response to fungi is the recognition of invading fungal pathogens via pattern recognition receptors (PRRs), which are located mainly in host cells of the innate immune system (Netea and Marodi, 2010). All types of PRRs i) recognize PAMPs, that are vital for the survival of the microorganism; ii) are expressed constitutively in the host and detect the pathogens regardless of their life-cycle stage; iii) are germline encoded, nonclonal, expressed on all cells of a given type, and independent of immunologic memory (Netea and Marodi, 2010; Romani 2011). Overall, in response to fungal recognition, PRRs on phagocytes initiate downstream intracellular events that promote the activation of the immune system and the clearance of fungi, with the specific immune response generated depending on the cell type involved (Akira *et al.* 2006; Romani, 2011). The recognition of PAMPs is done by several series of PRRs, such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and the galectin family receptors (Taylor *et al.,* 2007; van der Meer *et al.*, 2010; Mora-Montes *et al.,* 2011; Romani, 2011; Netea *et al.* 2015), as represented in Figure 3.

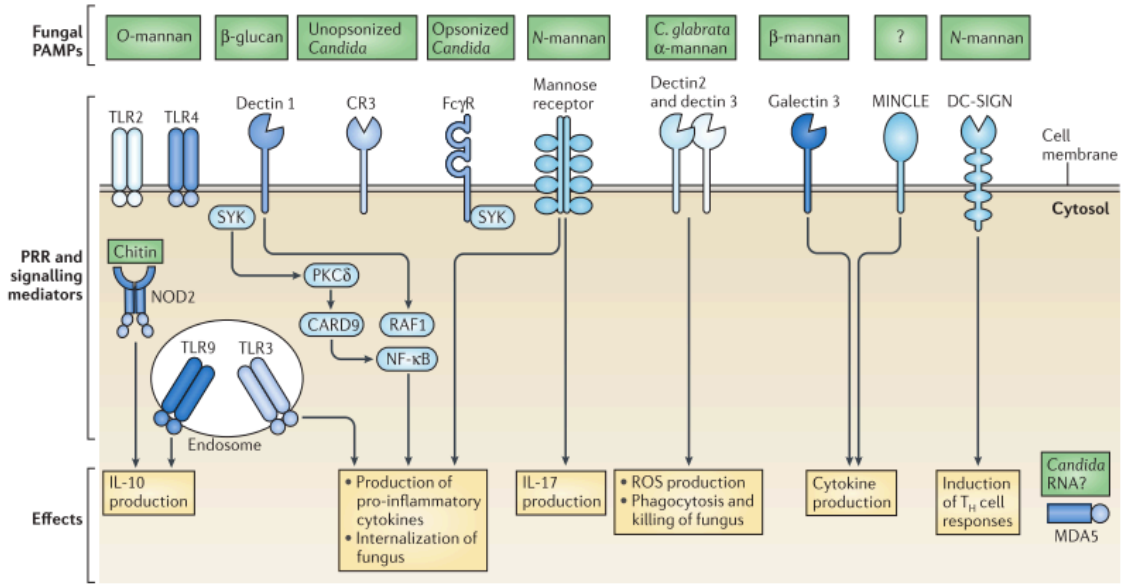

Figure 3: Signalling pathways in innate recognition of fungi. PAMPs and damage-associated molecular patterns (DAMPs) that are present during fungal infections are recognized by PRRs. Together, these signalling pathways induce the secretion of cytokines and chemokines and initiate phagocytosis to clear *Candida* infections (Adapted from Netea 2015).

5.2.1 Fungal sensing by TLRs

TLRs are cell-membrane-associated (TLR1, TLR2, TLR4, TLR5 and TLR6) or intracellular (TLR3, TLR7, TLR8 and TLR9) receptors that have been implicated in the recognition of fungal components. Is difficult to precise which is the exact molecular nature of fungal PAMPs that activate specific TLRs, due to the often collaborative mechanism of TLR recognition and the plasticity of the fungal cell wall. Membrane-bound TLRs, such as TLR2, TLR4 and TLR6, are thought to mainly recognize mannoprotein constituents of the fungal cell wall: TLR2 recognizes phospholipomannan, TLR4 recognizes O-linked mannans, and TLR6 is involved in the recognition of zymosan, which is comprised of β-1,3-glucan and mannans and TLR9 detects fungal DNA (Romani, 2011; Netea *et al.* 2015). It has also become apparent that the intracellular receptors that recognize cytoplasmic nucleic acids, namely TLR3 and TLR9, may also have a role in anti-*Candida* host defence (Netea *et al.,* 2015). Whereas fungal PAMPs are recognized by a number of TLRs these might not the primary receptors driving pathogen engulfment (Bourgeois and Kuchler, 2012).

5.2.2 Fungal recognition by CLRs

CLRs are the most important family of innate receptors in the recognition of *Candida* species. CLRs are mainly membrane-bound receptors that recognize polysaccharide structures of the *Candida* cell wall. Dectin-1 recognizes β-1,3-glucans, whereas the macrophage mannose receptor (MR) and DC-SIGN senses N-linked mannans (Netea *et al.* 2008; Romani, 2011; Netea *et al.* 2015), and Dectin-2 usually recognizes α-mannans. Several studies demonstrated that mice with deficiencies in CLRs, namely Dectin-1, Dectin-2, and MINCLE, or CARD9 are more susceptible than wild-type mice to disseminated invasive candidiasis, which is associated with reduced T helper Th17 cell frequency (Bishu *et al.,* 2014).

Recognition of β-glucan

Different authors suggest that β-glucans are shielded from immune recognition by the mannoproteins in *C. albicans* and that unmasking these polysaccharides results in the induction of proinflammatory responses (Davies *et al.*, 2004; Netea *et al.,* 2015). The most well-studied β-glucan receptor is Dectin-1 (also known as CLEC7A), expressed mainly on monocytes and macrophages, but also in dendritic cells and neutrophils (Brown *et al.,* 2002; Brown and Gordon,

2001). Recognition of β-glucans by Dectin-1 can trigger phagocytosis of fungal pathogens and protective inflammatory responses (Brown, 2006). Activation of this receptor has been shown to be immunostimulatory, stimulating reactive oxygen species (ROS) production in murine cells (Wellington *et al.* 2009). Dectin-1 signals via a novel hemITAM motif that becomes phosphorylated by Src family kinases on receptor engagement, as well as through the RAF1 kinase signalling pathway. This allows recruitment and activation of the spleen tyrosine kinase (Syk), which then couples to downstream pathways, including those leading to production of ROS and, via the adaptor CARD9, to the activation of NF-kB. The latter, together with transcription factors activated by MAP kinases downstream of Syk, regulates the expression of innate response genes, including those encoding co-stimulatory molecules and proinflammatory cytokines and chemokines (Brown *et al.,* 2002; Hernanz-Falcón *et al.,* 2009; Netea *et al.,* 2015).

In addition to inducing direct cellular activation, engagement of Dectin-1 intensifies responses to TLR2 and TLR4 ligation, TLRs that recognize mannan-containing structures (Netea *et al.,* 2015)

Recognition of mannans

Both mannans and mannoproteins from the *C. albicans* cell wall have important immunostimulatory activities, ranging from stimulation of cytokine production to induction of DC maturation and T-cell mediated immunity. Mannoproteins induce mainly Th1 type cytokine profiles, which have protective effects against disseminated *C. albicans* infections (Netea *et al.,* 2008). Mannans and mannoproteins are recognized by several CLRs, including the mannose receptor, Dectin-2 (CLEC6A), dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN; also known as CD209) and MINCLE (also known as CLEC4E) (Netea *et al.,* 2008; Netea and Marodi, 2010; Netea *et al.,* 2015). The mannose receptor is primarily expressed on macrophages and recognizes *N*-mannan (Netea *et al.,* 2008). Dectin 2, which is mainly expressed on dendritic cells, macrophages and neutrophils and generally recognizes α-mannan. In addition to its role in modulating Th17-type cell responses, Dectin-2 has been associated with the production of ROS and with phagocytosis and elimination of *C. glabrata* (Netea *et al.,* 2008; Netea *et al.,* 2015). Dectin-2 has been reported to form heterodimers with Dectin-3, and owing to its short intracytoplasmic tail, Dectin-2 must interact with the FcγR to induce intracellular signals. Dectin-2 is encoded by a different gene cluster to the β-glucan receptor Dectin-1 and does not recognize β-glucans (Ifrim *et al.,* 2014; Netea *et al.,* 2015).

Mincle is a CLR mainly expressed on macrophages/monocytes, myeloid dendritic cells, and neutrophils. It recognizes carbohydrate structures containing α-mannans and is responsible for inducing protective responses, largely by stimulating TNF-α production (Netea *el at.,* 2015). Like Dectin-2, MINCLE signalizes after dimerisation with the FcγR adaptor protein (Naglik, 2014).

DC-SIGN is present especially on DCs but also on macrophages and recognizes *Candida* N-linked mannan. Its activation promotes adaptive immune responses by inducing the expression of cytokines that drive the activation of Th cells (Netea *et al.,* 2008; Netea *et al.,* 2015).

5.2.3 Fungal recognition by NLRs

NLRs are cytoplasmic receptors that achieve several important biological functions, including the recognition of bacterial peptidoglycans, antigen processing and presentation, activation of the inflammasome, and are also implicated in sensing fungi. NOD1 and NOD2 are two well-characterized NLRs that are involved in the recognition of gram negative and gram positive bacteria components and both NOD1 and NOD2 have been shown to be important for the response to *Aspergillus fumigatus* keratitis (Zhang *et al*., 2014; Wu *et al*., 2015). The role of these NLRs to *Candida* infection is not clear, since some authors stated that these receptors were not associated with *Candida* recognition (Van der Graaf *et al*., 2006), while others reported recognition and involvement of chitin-mediated responses, especially the production of IL-10 in the context of interaction between NOD2, MR and TLR9 (Wagener *et al.,* 2014).

Other NLRs such as Nlrp1, Nlrp3 and Nlrp4 oligomerize to form inflammasome complexes. Inflammasome oligomerization leads to the cleavage and activation of Caspase-1, which promotes the processing and secretion of the proinflammatory cytokines IL-1β and IL-18 (Netea *et al.* 2008; Netea and Marodi, 2010; Romani, 2011). Several NRL have been associated with a protective response to fungi (Veerdonk *et al.,* 2015). Both *S. cerevisiae* and *C. albicans* are able to trigger Nrlp3 activation leading to IL-1β production and the induction of Th1 and Th17 cell differentiation (Kumar et al., 2009). While NLRP3 activation is critical for the control of both systemic and mucosal *C. albicans* infections Nrlc4 is important in limiting mucosal candidiasis (Tomalka *et al*., 2011). Nrlp10 signalling affected mostly the adaptive response to *C. albicans* (Joly *et al*., 2012).

5.2.4 Fungal sensing by other PRRs

Complement receptor 3 (CR3) is a widely expressed β2-integrin that recognizes several endogenous and exogenous ligands, pathogens that have been opsonized by iC3b (the inactivated form of complement component C3b) and carbohydrates, including β-glucans. This receptor has two binding sites, one for iC3b and a different one for β-glucans, and is mainly involved in the recognition of β-glucans by neutrophils and macrophages. Recognition by CR3 induces phagocytosis but does not trigger protective host responses, such as the respiratory burst, and can repress proinflammatory signals (Netea *et al.,* 2008; Netea *et al.,* 2015).

Galectin-3 is one of more than 15 members of the β-galactoside-binding lectin superfamily involved in the recognition of β-1,2-mannosides β–mannosides (Chen *et al.*, 2015). Murine macrophages can bind, and directly kill, *C. albicans* that harbour β-1,2-mannosides, but not *S. cerevisiae*, which lack those residues, through galectin-3. In response to β-1,2-mannoside-binding, galectin-3 induces a protective antifungal response in mouse macrophages through secretion of TNF-α in collaboration with TLR2 (Jouault *et al.* 2006; Kohatsu *et al.*, 2006; Gazendam *et al.,* 2014).

The scavenger receptors are defined as a family of molecules that share the ability to bind polyanionic ligands of both pathogen and self origin. CD36 and SCARF1 have also been reported to recognize β-glucans of *Candida* species cell wall and to have a role in the protective response to fungi (Means *et al.,* 2009; Means, 2010).

6. *Candida* invasion and host cell associated mechanisms of defence

After the initial recognition of fungal PAMPs by the various families of PRRs, a chain of effector mechanisms is initiated that ultimately leads to the clearance of the invading fungal pathogen. One of the key steps in *Candida* pathogenesis is adherence, followed by invasion, usually into epithelial cell layers that also display a role in controlling the commensal state of *Candida* by producing β-defensins, which have potent antifungal activity (Netea *et al.,* 2015). *C. albicans,* can invade host cells via two mechanisms: induced endocytosis by host cells and active penetration by *C. albicans* hyphae (Wächtler *et al.*, 2011; Wächtler *et al.,* 2012). Induced endocytosis seems to be important at the early stages of invasion (Phan *et al.,* 2007; Wächtler *et al.*, 2011; Zhu *et al.,* 2012). In *C. albicans*, active penetration of host cells by hyphae result of a combination of physical forces

exerted by the extending filaments, the secretion of hydrolytic enzymes and yet unknown damaging factors, which finally leads to disruption of the host cell membranes (Wächtler *et al.,* 2012). Secreted aspartyl proteinases (Sap) are the most well studied hydrolytic enzymes produced by *C. albicans* during infection and are required for full virulence in models of mucosal and disseminated infection (Schaller *et al.*, 2002; Naglik *et al.,* 2003; Schaller *et al.,* 2004; Moyes *et al.,* 2010). Conversely, *C. glabrata* does not form hyphae nor produce Saps, but still can invade and be found within host tissues. A possible route to breach natural barriers would be via trauma, catheters, surgery or parenteral nutrition (Perlroth *et al.*, 2007). However, even in the absence of such breaches, *C. glabrata* invades into deeper tissues and readily disseminates in a chicken embryo model of fungal infection, and superficially invades the mucosal compartment in a 3-D model of oral mucosa (Li *et al.*, 2007; Jacobsen *et al.*, 2011). The strategy of invasion remains unclear although *S. cerevisiae* is known to form agar-invasive pseudohyphae under *in vitro* starvation conditions, and one report even described psedohyphae formation by *C. glabrata in vitro* (Gimeno *et al.*, 1992; Csank and Haynes, 2000). However, *in vivo*, *Candida* entry may rely on endocytosis instead of active penetration, with close to no host cell damage. Probably because of low host tissue damage, the cytokine profile of epithelia infected with *C. glabrata* differs dramatically from that of *C. albicans*-infected cells (Schaller *et al.*, 2002; Li *et al.,* 2007; Jacobsen *et al.*, 2011).

Once *Candida* has penetrated the host tissues it will first encounter the tissue resident macrophages (Netea *et al.,* 2015), key effector cells in antifungal defence. These are phagocytic immune cells, derived from monocyte differentiation, that are involved in the first line of defence during microbial invasion, produce inflammatory cytokines and chemokines that recruit and activate other immune cells at the site of infection (Netea *et al.,* 2015). Macrophages express a wide range of different opsonic and non-opsonic receptors able to detect non-self particles. Opsonic receptors, such as the Fc receptor or complement receptor families are able to recognize particles coated (opsonized) with antibody or complement proteins (Johnston and May, 2013). In contrast, non-opsonic receptors are PRRs that directly sense the surface of microorganisms (Gilbert *et al.,* 2014).

Upon pathogen engulfment, the macrophage must then digest it, now confined into a phagosome (digesting vesicle). To complete the digestion, the phagosome must mature via the fusion of early and late stage endosomes and ultimately fuse with the lysosomes, generating a full destructive phagolysosome (Kinchen and Ravichandran, 2008; Seider *et al.,* 2011). Acidification of the phagosome occurs in two distinct stages. First a primary acidification that results in a relatively

small drop in pH, and subsequently V-type (vacuolar) ATPase proton pumps driven by hydrolysis of ATP are trafficked to the phagosome at late stages. Once the pH is suitably reduced, acid-dependent proteases, such as cathepsin D, are activated to degrade the pathogen (Kinchen and Ravichandran 2008). Furthermore, during phagosome biogenesis a battery of ROS such as peroxide, superoxide anions and hydroxyl radicals, reactive nitrogen species, and antimicrobial peptides are transported into the organelle (Segal *et al*., 2005; Seider *et al.,* 2011; Mayer *et al.* 2013). Several studies suggest that live *Candida* (*C. albicans* and also *C. glabrata*) suppresses the production of ROS by phagocytes. Suppression of ROS production does not occur with *S. cerevisiae,* suggesting that it may be an important factor in the pathogenesis of *Candida* infection. Suppression of ROS production appears to override stimulatory signals from the cell wall, including those induced by β-1,3-glucan (Wellington *et al*., 2009; Seider *et al.,* 2011).

C. albicans and *C. glabrata* interfere with macrophage phagosome maturation to avoid acidification and limit the hydrolytic attack (Fernandez-Arenas *et al.,* 2009; Seider *et al.,* 2011). However, neither the mechanisms to limit phagosome maturation nor the relative contribution of these to fungal survival are known. It is known, nevertheless, that blocking phagosome–lysosome maturation plays an important role for many intracellular pathogens (Gilbert *et al.* 2014).

In vitro experiments suggest that *C. albicans* can germinate within macrophages to causing macrophage membrane rupture, and eventually lysis, allowing *C. albicans* to escape. Intriguingly, this macrophage lysis may be a result of pyroptosis instead of physical membrane rupture (Calderone and Sturtevant 1994; Vazquez-Torres and Balish 1997; Wellington *et al.,* 2012). *Candida* species that do not filament, such as *C. glabrata*, are not always less virulent, indicating that other mechanisms exist to allow survival, replication, and escape from macrophages after being phagocytosed. Some authors defend that *C. glabrata* attracts and infect macrophages, and use them as "trojan horses", to hide from immune surveillance and to spread to the organs, similarly to what is seen in *Cryptococcus neoformans* infections (Charlier *et al.,* 2009; Brunke and Hube, 2013). Degradation and recycling of endogenous cellular components (autophagy) seem to be another intracellular survival strategy of *C. glabrata* within macrophages (Roetzer *et al.,* 2010).

Other cells shown to be essential for the host response against *Candida* species are DCs. DCs can also ingest and kill *Candida* cells, however, they are less efficient than macrophages at fungal killing. The ability of a given DC subset to respond differentially and to activate distinct intracellular signalling pathways following the ligation of different PRRs, confers unexpected plasticity to the DC to shaping T cell responses to infection and following vaccination. DCs are important for processing

and presenting fungal antigens for the activation of Th cell responses (Netea *et al.,* 2015). Of the diverse T-cell subsets, Th1, Th17, and regulatory T cells (Treg) have been shown to be important in controlling *C. albicans* infection or pathology resulting from infection. Through the production of the cytokine IFN-γ Th1 cells are instrumental in the optimal activation of phagocytes at sites of infection (Blanco and Garcia, 2008, Romani, 2011), however, it is also clear that patients with genetic defects in the IL-12, IL-23 and IFN-γ pathways do not have increased susceptibility to mucosal candidiasis (Puel *et al.,* 2012).

Not only Th1, but also Th17 type responses are important for host defence against *Candida* species. In accordance, human *Candida*-specific Th cells were found to produce a combination of IL-17 and IFN-γ (Zielinski *et al.,* 2012; Netea *et al.,* 2015).

Th17 cell activation occurs in fungal infections, mainly through the SYK–CARD9, signaling pathways in DCs and macrophages resulting in the production of cytokines that induce neutrophil recruitment and activation, and are responsible for the activation of epithelial cells and the release of antifungal β-defensins, often in a cooperative manner. The importance of Th17 immunity in response to *C. albicans* infections was first shown in murine studies of both systemic and mucosal candidiasis (Huang *et al*., 2004; LeibundGut-Landmann *et al*., 2007; Conti *et al*., 2009). However, human genetic deficiencies have indicated a role for the Dectin-1/CARD9, STAT3 and Th17 cell pathways in protection against mucosal infections but not against systemic candidiasis (Netea *et al*., 2015).

It is now well established that a successful host immune response to *C. albicans* infection relies on the concerted and balanced action of Th1, Th17, and also Treg. Th1 and Th17 cells are undoubtedly important in host resistance to the different forms of candidiasis, however, their action must be counterbalanced in order to avoid harmful host inflammation. Studies in the murine model have shown that Treg are important in limiting the inflammatory immune response to *C. albicans* in systemic and mucosal candidiasis (De Luca *et al.,* 2007; Netea *et al.,* 2004), but their role might exceed a counterinflammatory function, since Treg might promote, rather than suppress, Th17 cell differentiation (Pandiyan *et al.,* 2011). Indeed, in murine models of systemic candidiasis Treg depletion have shown to increase mice resistance to infection, while in a mucosal model of infection mice Treg depletion rendered mice more susceptible (Netea *et al*., 2004; Whibley *et al*., 2014; Whibley and Gaffen, 2014), what could provide an explanation for why patients with Treg defects are prone to mucosal *C. albicans* infections.

In contrast to the reported protective Th1 and Th17-type immune responses that are important for a protective host response to *Candida* species (Carvalho *et al.,* 2012), cytokines associated with Th2-type immunity have been shown to have contradicting roles. Adoptive transfer of IL-4, and also IL-10-deficient T cells in bone-marrow irradiated wild-type mice increased their resistance to systemic candidiasis (Mencacci *et al.,* 2001). In accordance, IL-10-depleted mice showed greater resistance to candidiasis (Vazquez-Torres *et al.*, 1999; Tavares *et al.*, 2000), suggesting a negative role for these cytokines. Conversely, early IL-10 administration was protective in IL-12 p40-deficient mice and endogenous IL-4 was required for the development of protective Th1 responses to *Candida albicans* (Mencacci *et al.*, 1998)

7. Objectives and outline of the thesis

Our research group described in 2006 a new fungal species, *C. bracarensis*, phenotypic and genetically related with *C. glabrata* and, to a lesser extent, with *S. cerevisiae* (Correia *et al.*, 2006). The incidence of this new species has been increasing worldwide, being considered as an emerging pathogen. However, little is known about the characteristics of *C. bracarensis* as a pathogen and its interaction with the host.

The main objectives of this work are: i) to evaluate *C. bracarensis*, *C. glabrata*, and *S. cerevisiae* resistance to several oxidative and cell wall stressors; ii) to analyze cell wall composition/structure of these yeats; iii) to characterize the interaction of *C. bracarensis*, *C. glabrata*, and *S. cerevisiae* with cells of the host innate immune system, such as macrophages and dendritic cells;

This dissertation is organized in four chapters:

Chapter 1 consists of a general introduction. **Chapter 2** contains the methodology used to carry out the experiments. **Chapter 3** describes the main results obtained. Here, at first, a phenotypic characterization of several *C. bracarensis*, *C. glabrata* and *S. cerevisiae* strains is presented, concerning resistance to oxidative and cell wall stress agents and the composition/structure of yeasts cell wall. Then, data on the phagocytosis of yeasts by murine and human macrophages, as well as reactive oxygen species production by these phagocytes is shown. Finnally, the results on the interaction of yeasts with macrophages and dendritic cells and the elicited inflammatory response are presented. **Chapter 4** consists of a general discussion of the experimental results and the main conclusions, and at last, future perspectives are also suggested.

The work presented in this dissertation was developed at Laboratory of Immunology, Institute of Biomedical Sciences and Institute for Molecular and Cellular Biology, University of Porto, and also at Centre of Molecular and Environmental Biology, Biology Department of University of Minho. Cell wall characterization studies were performed at Chemistry Department, University of Aveiro.

Chapter 2

Material and Methods

1. Phenotypic and functional characterization of *C. bracarensis, C. glabrata* and *S. cerevisiae* strains

1.1 Fungal strains and yeast cell cultures

Five strains of each selected species (*C. bracarensis, C. glabrata and S. cerevisiae*) were analyzed, in a total of fifteen yeast strains, (Table 1).

Table 1: *C. bracarensis, C. glabrata and S. cerevisiae* strains used in this study

Specie	Strain	Source	Origin
Candida bracarensis	153M^{T}/CBS10154T	Vaginal swab	Portugal
	246188	Vaginal swab	Portugal
	445L	Rectum	France
	CL7030(L08/524)	NS	Spain
	NCYC 3133	Blood	United Kingdom
Candida glabrata	CBS 138	Faeces	NS
	CIPO23	Respiratory fluid	Portugal
	CIPO44	Faeces	Portugal
	CIPO88	Vaginal swab	Portugal
	Hemo1117	Blood	Portugal
Saccharomyces cerevisiae	L557/ S288C	Baker	NS
	L559	Mucosa	Portugal
	L560	Mucosa	Portugal
	CIPO12	Faeces	Portugal
	CIPO56	Respiratory fluid	Portugal
Candida albicans	SC5314 /ATCC MYA-2876	NS	United Kingdom

NS- not specified

All strains were maintained as frozen stocks in 30% glycerol at −80°C. Viable cells were obtained by cultivation on solid yeast extract-peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% glucose, 2% agar). Isolates were then grown in liquid YPD medium at 37°C and 140 rpm in a shaking incubator (3031-GFL, Germany) to late exponential growth (14-16 hours), recovered by

centrifugation (10 minutes at 4500 g, 4°C) and washed twice in sterile PBS buffer. Cells were counted with a hemocytometer and brought to desired final concentration.

Inocula were always confirmed by colony forming unit (CFU) counts on YPD solid medium for up to 24 hours at 30/37°C.

In selected experiments for cell wall characterization (Chapter 3), yeast cells were grown in liquid Sabouraud medium (2% peptone, 2% glucose) at 37°C and 140 rpm to late exponential growth (16 hours). The use of Sabouraud instead of YPD medium had the aim of avoiding contamination of yeast cells by yeast extract, which could influence the results. Cells were recovered by centrifugation (20 minutes at 6400 g, 4ºC), washed three times with sterile distilled water, frozen, and freeze-dried (Christ Alpha 2-4, B. Braun, Germany).

For preparation of heat-killed yeast cells, cells were grown as described before and incubated at 70˚C for 30 minutes in sterile PBS.

To fix yeast cells, these were incubated for 10 minutes in formol/ethanol (1:9) and washed three times with PBS for complete removal of formol/ethanol.

1.2 Isolate identification by ITS-DNA sequencing

The identity of all *C. glabrata*, *C. bracarensis,* and *S. cerevisiae* strains was confirmed by ITS-DNA sequencing, as follows:

1.2.1 PCR amplification

Colony-polymerase chain reaction (PCR) is a simple and fast methodology that uses directly the colony in the PCR tube to obtain the DNA template. To perform this technique, a protocol set at our laboratory was used (Vaz *et al.*, 2011). In brief, a single colony was picked and transferred to a PCR microtube. For DNA release, the cells were lysed by thermal shock during 90 seconds in the microwave, and the microtubes were immediately placed on the ice to inhibit DNA degradation. Finally, a total volume of 24 µl of the PCR reaction mix was added. The specific primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used. The PCR reaction mix was performed by combining 1× PCR Buffer (20 mM TrisHCl [pH 8.4], 50 mM KCl), 0.2mM of each of the four dNTPs, 2 mM $MgCl_2$, 1 U of Taq polymerase and the 0.2 µM of each primer in a 10 µl final volume. The samples were amplified in a UNO II thermocycler (Biometra, Germany) and the PCR program consisted of an initial denaturation step of 6 min at 95°C, followed by 35 cycles of 20 s at 95°C, 20 s at 53°C, 1 min at 72°C, and a final extension step of 5 min at 72°C.

DNA amplification was confirmed by electrophoresis, using an agarose gel 1.5% and Tris-acetate-EDTA (TAE) buffer. The electrophoresis was performed at 100mV during 45 min. The gel was stained with Midori Green (LabGene, Suisse) for at least 30 min in agitation and visualized under UV-light on a UV transilluminator (Vilber Lourmat TF-20M, France).

1.2.2 Purification of PCR products

After amplification, a total of 35 µl of PCR product was used for DNA purification. 175µl of cold ethanol 95% and 70 µl of $MgCl_2$ (2 mM) were added to the PCR product and the mix was mixed. The mixture obtained was incubated at -20°C by 20 min and centrifuged for 20 min at 14000 g, 4°C. The supernatant was discarded and 250µl of 70% ethanol were added to the pellet. After a new centrifugation step the pellet was discarded. This step was performed twice. Finnaly, the pellet was dryed at room temperature (RT) and 25 µl of ultra pure water (pre-heated at 50-60°C) were added.

1.2.3 Sequencing and data analysis

After purification, the PCR products were sent to DNA sequencing by Eurofins (Portugal) (http://www.eurofins.pt), the electropherograms were analyzed through the CodonCode Aligner program, and the sequences were submitted to a BLAST search on NCBI's database GenBank.

1.3 SAP production

Overnight yeast cultures were washed and plated (10 µl of cellular suspension OD_{640}=0,5) on solid YCB-BSA (1,17% YCB; 0,01% yeast extract; 0,2% BSA, pH=5). Plates were incubated at 30ºC and 37ºC during 10 days. After the incubation period, plates were stained with stain solution (25% methanol, 10% acetic acid, 0,1% amido black) for 15 minutes and were then washed several times with destain solution (25% methanol, 10% acetic acid). The determination of proteolytic activity was performed by observation of the presence or absence of an opaque inhibition halo around the colonies (Price *et al.,* 1982). *C. albicans* was used as a positive control.

1.4 Filamentation test

Filamentation was induced by plating 10 µl of an overnight cellular suspension (OD_{640}=0,5) on solid Spider medium (1% DIFCO Nutrient Broth; 1% Manitol; 0,2% dibasic potassium phosphate),

solid Slad medium (0,17% YNB; 2% dextrose; 50 μM ammonium sulfate) or solid YPD supplemented with 10% fetal bovine serum (FBS, Invitrogene, USA). The plates were incubated at 37ºC during 10 days.

1.5 Susceptibility assay

The sensitivity of the studied strains to various stress conditions was tested using overnight yeast cultures, washed and diluted to OD_{640}= 0,5 in YPD fresh medium. Drop tests were performed by spotting 5 µl of the serially diluted cell suspensions onto YPD plates supplemented with following compounds: CongoRed (70 µl/ml and 250 µl/ml); caffeine (10 mM and 20 mM); caspofungin (75 ng/ml, 120 ng/ml and 180 ng/ml); Calcofluor White (50 µg/ml and 250 µg/ml); SDS (0,02%, 0,05% and 0,15%); NaCl (1M and 2M). to test the susceptibility to H_2O_2, the overnight grown cultures were incubated with 20 mM or 50 mM of H_2O_2 and were kept under the same growing conditions during 3 hours. Plates were incubated for 48 h at 30ºC and 37ºC before observation.

2. Characterization of cell wall composition/structure of *C. bracarensis, C. glabrata* and *S. cerevisiae*

2.1 Extraction of yeast polysaccharides

An aqueous suspension of yeast cells was submitted to 15 min of sonication, and the solubilized material was separated from the insoluble residue by centrifugation at 4°C and 5500 g during 20 min.

The yeast cells residues were boiled with 80% ethanol during 10 min in order to prepare the alcohol insoluble residues (AIR).

Each AIR sample was submitted to a hot water extraction during 15 min at 100°C with an AIR/water ratio of 1:16 (w/v), and the insoluble material was separated from the soluble one by centrifugation. The extracted material was dialyzed against water with 12–14 kDa molecular weight cut off membranes.

2.1.1 Neutral sugar analysis

Sugar composition of the polysaccharides was determined by gas chromatography-flame ionization detection (GC-FID) and quantified using 2-deoxyglucose as internal standard (Blakeney *et al.,* 1983). Monosaccharides were released from polysaccharides (1–2 mg of sample) by pre-hydrolysis in 0.2 ml of 72% H_2SO_4 for 3 h at RT followed by 2.5 h hydrolysis in 1M H_2SO_4 at 100°C (Selvendran, March, & Ring, 1979).

Monosaccharides were reduced with $NaBH_4$ (15% in 3 M NH_3) during 1 h at 30°C and then acetylated, with acetic anhydride (3 ml) and 1-methylimidazole (450 μl), during 30 min at 30°C. The alditol acetates were separated by liquid-liquid extraction with water and dichloromethane and, after evaporation of the organic solvent, they were solubilized in 50 μl anhydrous acetone and analysed in a GC-FID Perkin Elmer-Clarus 400 with a capillary column DB-225 (30 m length, 0.25 mm inner diameter and 0.15 μm film thickness). The oven temperature program was as follows: 200°C to 220°C at a rate of 40°C/min (7 min), increasing to 230°C at a rate of 20°C/min (1 min). The temperature of the injector was 220°C and the detector was at 230°C. Hydrogen was used as carrier gas at a flow rate of 1.7 ml/min (Bastos *et al.*, 2015; Pinto *et al.*, 2015).

2.1.2 Glycosidic-linkage analysis

The glycosidic-linkage composition was determined by gas chromatography-mass spectrometry (GC-MS) of partially methylated alditol acetates (Ciucanu & Kerek, 1984; Nunes & Coimbra, 2002). The samples (1 mg) were dissolved in 1 ml of anhydrous dimethylsulfoxide (DMSO), and then powdered NaOH (40 mg) was added under an argon atmosphere. The samples were methylated with CH_3I (80 μl) during 20 min with stirring, followed by a second addition of 80 μl CH_3I and stirring for another 20 min. $CHCl_3/CH_3OH$ (1:1, 3 ml) was added and the solution was dialyzed (membrane with a pore diameter of 12–14 kDa) against 3 lots (1000 ml) of 50% ethanol. The dialysate was evaporated to dryness and the material was remethylated using the same procedure. The remethylated material was hydrolysed with 2 M trifluoracetic acid (TFA) (500 μl) at 120°C for 1 h, and then reduced and acetylated as previously described for sugar analysis, using $NaBD_4$ instead of $NaBH_4$. The partially methylated alditol acetates were separated and analysed by GC-qMS using an Agilent Technologies 6890 N Network. The GC was equipped with a DB-1 (J&W Scientific, Folsom, CA, USA) capillary column (30 m length, 0.25 mm of internal diameter, and 0.15 μm of film thickness). The samples were injected in "split" mode using an injector temperature of 220°C, during 5 min. The temperature program used was as follows: the initial

temperature was 50C, with a linear increase of 8°C/min until 140°C (5 min), followed by a linear increase of 0.5°C/min until 150°C and finally by a linear increase of 40°C/min until 250°C (1 min). The helium carrier gas had a flow rate of 1.7 ml/min and a column head pressure of 14.4 psi. The GC was connected to an Agilent 5973 mass quadrupole selective detector operating with an electron impact mode at 70 eV and scanning the range m/z 40–500, 1 s cycle, in a full scan mode acquisition (Bastos *et al.* 2015; Pinto *et al*. 2015).

2.2 Determination of β -1,3 glucan exposure

The exposition of β-1,3-glucans on the cell wall surface of *C. bracarensis, C. glabrata,* and *S. cerevisiae* was evaluated by flow cytometry analysis and immunofluorescence confocal microscopy upon incubation with an anti-β-1,3-glucan monoclonal antibody (mAb). $1x10^6$ cells were incubated with anti-β-1,3 glucan mAb (Biosupplies) during 20 min at 4°C, and were stained with a anti-mouse IgG conjugated with Alexa Fluor 488 (Molecular Probes, Invitrogen, USA) for 20 min at 4°C in the dark. Cells were then washed with PBS, resuspended in FACS buffer (PBS; 1% BSA; 2 mM sodium azide), and analysed by flow cytometry (EPICS XL-MCL, Beckman-Coulter Corporation Miami, EUA). To unmask β-1,3-glucans, since these are usually localized in the inner layer of the cell wall and limitedly accessed (Brown *et al.,* 2014) cells were heated-killed (HK), as previously described prior to staining. Flow cytometry data were analysed by using FlowJo software (version 0.7- Treestar, Ashland, OR, USA).

For immunofluorescence confocal analysis, stained yeasts were fixed with 4% paraformaldehyde (10 min, 37˚C), washed with PBS, and finally were mounted in coverslips with mounting medium (ibidi, Germany). The exposure of β-1,3-glucans was analysed under a laser scanning confocal microscope (Leica TCS SP5 II running LAS AF 2.6 software; Leica Microsystems, Germany) using a 60x/1.40 numerical aperture (NA) oil immersion objective. Confocal microscopy images were analysed using FIJI- ImageJ software 2.00 (NIH-USA).

2.3 Quantification of yeast cell wall porosity: Polycations assay

Overnight grown yeast cells were washed three times with distilled water. Yeasts ($1x10^8$) were incubated at 30 °C for 30 min at 250 rpm in 1 ml 10mM-Tris-HCl, pH 7.4 containing 10 μg poly-L-Lysine (50 kDa) or 10 μg DEAE-dextran. As a control, cells were incubated in buffer without polycations. After incubation, the cells were centrifuged at 10000g for 10 min and the

supernatant's absorvance (A_{260nm}) was measured. Relative porosity was defined as (A_{260nm} DEAE-dextran – A_{260nm} buffer) × 100/(A_{260nm} poly-L-Lysine – $A_{260nm\ buffer}$) (de Nobel *et al,.* 1990).

3. Interaction of yeast with innate immune cells

3.1 Macrophage culture

The murine macrophage-like cell line RAW 246.7 was cultured in complete Dulbecco's modified Eagle's medium (cDMEM) (Sigma Aldrich, USA) supplemented with 10% heat-inactivated FBS (invitrogen), 1% glutamine, 1% Penicilin-streptomycin, and 1% HEPES (all from Sigma Aldrich), at 37 °C in a 5% CO_2 humidified atmosphere. Sub-cultures were performed into new cell-culture flasks every 3 days. After confluent growth, macrophage cells were recovered and washed. Cell concentration and viability were determined by Trypan blue (Sigma Aldrich) exclusion counting with a hemocytometer, and resuspended in cDMEM to the desired final concentration.

3.2 Human monocyte-derived macrophages

Human peripheral blood mononuclear cells (PBMC) were obtained from buffy coats donated by healthy volunteers by gradient centrifugation using Histopaque-1077 (Sigma-Aldrich), according to manufacturer's instructions. Then, monocytes were purified by magnetic cell sorting using CD14 microbeads (Miltenyi Biotec, Germany). To differentiate PBMCs into monocyte-derived macrophages (MDMs), cells were resuspended at $1x10^6$/ml in complete Roswell Park memorial institute (RPMI 1640) (Sigma Aldrich), supplemented with 10% autologous human serum, 1% penicillin-streptomycin, 1% HEPES buffer, 1% L-glutamine and 5 μM 2-mercaptoethanol (all from Sigma Aldrich), and platted in 24-well plates. Cells were incubated at 37°C with 5 % CO_2 during 7 days and the medium was renewed every three days. Adherent MDMs were then detached with 50 mM EDTA, washed and counted before plating in flat-bottom 24-well plates in cRPMI at the desired concentration.

3.3 Murine resident peritoneal macrophages

Mice (BALB/c) were anesthetized with isoflurane before being euthanized by cervical dislocation. Upon brief immersion in 70% ethanol, 5 ml of ice cold PBS were injected into the peritoneal cavity using a syringe and a 18 G needle. After gentle massage, the peritoneal fluid was collected. The recovered murine resident peritoneal macrophages (RPMs) were counted in a hemocytometrer and

seeded in a 96-well-plates (flat bottom).

3.4 Phagocytosis quantification assay

The phagocytosis of all yeast strains by RAW 246.7 and MDMs was analyzed by using a new methodology described for yeast phagocytosis analysis by FACS (Carneiro *et al.,* 2014). In brief, dead yeast cells (formol-ethanol fixed or heat killed), were incubated for 10 min with 5 μl of 1 μM Sytox Green (Invitrogen) at RT in the dark, washed twice with PBS to remove unbound dye and brought to the desired cell density in cDMEM. Macrophage cell suspensions (RAW, MDMs or RPMs) at final concentration of $5x10^5$ cells/ml were transferred to 24-well tissue culture plates. Cells were then incubated during three hours, at 37 °C and 5% CO_2, to allow macrophage adherence. Thereafter, phagocytic cells were washed twice with PBS to remove non-adherent cells and incubated with labeled yeast suspensions at a MOI of 1 Macrophage to 5 Yeasts (1M:5Y) for 15 and/or 30 min, at 37 °C and 5% CO_2. After incubation, wells were rinsed twice with warm PBS to remove unbound yeasts and plates were kept on ice to stop phagocytosis. Macrophages and associated yeasts were then incubated with 200 μl PBS and 10 μl Propidium iodide (PI; Sigma Aldrich), at a final concentration of 1 μg/ml, for 5 min at RT. Cells were washed with PBS to remove the excess of PI and lastly maintained in 300μl of FACS buffer. The cells were recovered by gently scrapping with insulin syringe plungers and analyzed by flow cytometry (EPICS XL-MCL, Beckman-Coulter Corporation Miami, EUA). Data were analysed by using FlowJo software (version 10.0.7). The percentage of yeasts interacting with macrophages was calculated from dot plot analysis of Sytox Green fluorescence intensity vs. PI fluorescence intensity, as described by Carneiro *et al.,* 2014.

In order to determine phagocytosis rates of viable yeasts, macrophages seeded in 24-well plates as described before, were infected with live yeasts labeled with 100 μg/ml fluorescein isothiocyanate (FITC; Sigma Aldrich at a MOI of 1M:5Y and co-incubated for 30 min at 37°C and 5% CO_2. Cells were stained with Tripan Blue (0,4 %) (Sigma Aldrich) right before analysis.

This double staining procedure was accomplished in order to quenching PI or TB over Sytox Green or FITC labelled yeasts, respectively. As neither, PI or TB enters in viable cells, the internalized yeast cells will retain the original unquenched fluorescence.

Alternatively, unstained live cells uptake was assessed by CFU counts after plating of the supernatants obtained by extensive washing of the yeast and macrophages co-cultures on YPD plates as follows: [(CFU of control well-CFU of test well)/ CFU of control well] x 100.

3.4.1 Blocking of phagocytosis receptors in RAW 246.7 and RPMs

To block Mannan Receptor and Dectin-1, RAW 246.7 and RPMs were incubated with 500 μg/ml soluble mannan (from *S. cerevisiae*) and 3 μg/ml anti-mouse Dectin-1 mAb (clone 218820; R&D Systems, USA) or 500 μg/ml laminarin (Sigma Aldrich), respectively, in RPMI without serum during 30 min, at 37 °C, prior to addiction of yeasts.

3.5 Determination of ROS production

ROS production was measured by using the Superoxide Detection Kit for flow cytometry (Enzo Life Sciences, USA). Viable yeast cultures were washed in PBS, ressuspended in cDMEM and co-incubated with macrophages (MOI of 1M:5Y) previously seeded in 24-well plates. Plates were incubated at 37°C and 5% CO_2 during 15, 30 and 60 min. As a positive control, macrophages were treated with 100 nM PMA (Sigma-Aldrich). After the incubation period, media were removed and cells were washed twice with Wash Buffer (supplied with the kit). Cells were detached from the tissue culture plates, as described above, collected and centrifuged for 5 min at 400 g at RT.
Lastly, the cell pellet was resuspended in 300 μl of superoxide staining solution during 30 min at 37°C in the dark. Samples were immediately analyzed by flow cytometry and data was analysed by using FlowJo software (version 10.0.7). Results are presented as the normalized percentage of ROS positive cells, considering that PMA stimulated 100% ROS production in macrophages.

3.6 Interaction between yeast and bone marrow derived dendritic cells (BMDC) and bone marrow macrophages (BMM)

3.6.1 Mice

Male BALB/c and C57BL/6 mice, 8 to 10 weeks old, were purchased from Charles River (Saint-Germain-sur-l'Arbresle, France) and kept under specific-pathogen-free conditions at the Animal Facility of the Instituto de Ciências Biomédicas Abel Salazar, Porto, Portugal. All procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS 123), the 86/609/EEC directive, and Portuguese rules (DL 129/92).

3.6.2 Differentiation of BMDC and BMM

Mice (BALB/c or C57BL/6) were anesthetized with isoflurane before being euthanized by cervical dislocation. Upon brief immersion in 70% ethanol, femurs and tibias were removed under aseptic conditions and flushed with cold Hank's balanced salt solution (HBSS) (Sigma Aldrich), in order to obtain undifferentiated bone marrow cells. The resulting cell suspension was passed through a 70 μm pore filter, centrifuged at 300 g, and resuspended in cRPMI medium. To remove fibroblasts or differentiated macrophages, cells were incubated on Petri dishes, during 5 hours at 37°C in a 5% CO_2 humidified atmosphere. Then, non-adherent cells were collected with warm cRPMI, counted in a hemocytometer, brought to a final concentration of $1x10^6$ cell/ml, in the appropriate medium, promptly seeded 5 ml of cell suspension per well in 6-well plates, and incubated at 37°C with 5 % CO2 for 7 days. cRPMI was supplemented with 20% J558-conditioned-supernatant (containing murine granulocyte-macrophage colony-stimulating factor (GM-CSF)) to differentiate cells into BMDC and medium was renewed every two days. To differentiate into BMM, cRPMI was supplemented with 10% L-cell conditioned medium (LCCM), the cultures were further supplemented at day 3 with 10% LCCM and medium was renewed at day 5.

Purity of derived DC's and macrophages was accessed upon staining with anti-mouse CD11c FITC-conjugated, (clone HL3) (BD Pharmingen), anti-mouse F4/80-PE-Cy5-conjugated, (clone BM8) (eBioscience), anti-mouse MHCII PE-conjugated (I-Ad/I-Ed, clone 2G9) (BD Pharmingen), anti-mouse CD86 PE-Cy7-conjugated, (clone GL1) mAb, and determined by flow cytometry.

3.6.3 Dectin- 1 blockage in BMDC and BMM

The blocking of the C-type lectin Dectin-1 in BMDC and BMM was done by incubation with 3 μg/ml anti-mouse Dectin-1 mAb (clone 218820; R&D systems), 1 hour prior to co-incubation with yeast cells.

3.6.4 Co-incubation of BMDC and BMM with *C. bracarensis, C. glabrata* and *S. cerevisiae* strains

After the 7-day-incubation period, BMDC and BMM were carefully recovered, washed with cRPMI, counted, and seeded in 96-well-plates (round and flat bottom, respectively).

Viable yeast cells were incubated with BMDC and BMM at a MOI of (1M:5Y) and (1M:2Y), and the plates were incubated during 24 hours at a 37°C and 5% CO_2. LPS (2 μg/ml, Sigma Aldrich) and unstimulated cells were used as positive and negative controls respectively. After the infection

period, the plates were centrifuged at 400 g during 10 min and supernatants were recovered and stored at -80°C until cytokine quantification.

3.6.5 Cytokine quantification by sandwich ELISA

The amount of IL-1β, IL-10, IL-12p40, IL-23 secreted by BMDC was determined by sandwich ELISA according to the manufacturer's instructions (Mouse IL-1β ELISA Ready-SET-Go!®; Mouse IL-12p40 ELISA Ready-SET-Go!®; Mouse IL-23 ELISA Ready-SET-Go!®, all from eBioscience, San Diego, EUA and Mouse IL-10 DuoSet ELISA Development System - R&D systems). All experiments were performed in triplicate. TNF-α levels were detected in the supernatants of both BMDCs and BMMs by ELISA using the Mouse TNF- α ELISA Ready-SET-Go!®, eBioscience.

3.6.6 Evaluation of bone marrow dendritic cells activation

For the assessment of cell surface activation markers, BMDCs were collected from the culture plates 24 h after yeast incubation, washed in FACS buffer and incubated with specific cell surface antibodies, anti-CD11c-FITC conjugated (clone HL3), anti-CD80-PE conjugated (clone 16-10A1), CD86-PECy7 conjugated (clone GL1) and MHC class II- PerCP conjugated (clone 2G9), all from eBiosciences.

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4. Isolation of mouse peritoneal cavity cells

4.1 Mice yeast infection and analysis of peritoneal cavity cells

To investigate the recruitment of myeloid cells at the site of yeast infection, sixteen BALB/c mice were infected intraperitoneally (i.p.) with 1 x 10^7cells of selected yeast strains in 0.4 ml PBS. Control mice were injected i.p. with PBS.

After 4, 6 or 72 hours of infection the animals were euthanized and 5 ml of ice cold PBS were injected into the peritoneal cavity using a syringe and a 18 G needle. After gentle massage, the peritoneal fluid was collected.

For cell surface marker staining, $1x10^6$ of peritoneal isolated cells were incubated with anti-mouse CD16/CD32 (Fc Block; 1:100) at 4°C for 15 minutes and then stained for 25 minutes with the appropriate antibody cocktails at previously optimized concentrations: anti-mouse GR-1 (Ly6C and Ly6G) FITC-conjugated, (clone R86-8C5; BD Pharmingen); anti-mouse integrin αM, Mac-1 α chain CD11b PE-conjugated, (clone M1/70; BD Pharmingen); anti-mouse F4/80 PE-Cy5-conjugated, (clone BM8; eBioscience); anti-mouse MHCII PE-conjugated, (clone 2G9; BD Pharmingen). After washing with FACS buffer, samples were analyzed by flow cytometry.

5. Statistical Analysis

All experiments were performed at least in triplicate ($n \geq 3$). All data were reported as means ± SD. Data were analysed by One-way ANOVA and Bonferroni post-Hoc test using GraphPad Prism (Version 5.00 for Windows) or using unpaired two-tailed t-test. Statistically significant results were defined as follows: $*P<0.05$, $**P<0.01$, and $***P<0.001$, ****P<0.0001.

Chapter 3

Results

1.Phenotypic characterization of *C. bracarensis*, *C. glabrata* and *S. cerevisiae* strains

1.1 Yeast identification

A yeast collection comprising 15 strains, five of each studied species, *C bracarensis*, *C glabrata* and *Saccharomyces cerevisiae*, was used here. Additionally, a well characterized *C. albicans* strain (SC5314) was used in specific experiments. In order to verify if all strains used in this study were properly identified, species identification was done by ITS-DNA sequencing followed by BLAST search of the ITS sequences in the NCBI GenBank database, thus confirming the identity of all strains used.

1.2 Sap production

The secretion of hydrolytic enzymes during infection represents one of the major virulence factors in *C. albicans*, since it facilitates invasion and colonization of tissues of the host by disrupting host membranes (Naglik *et al.,* 2003). Among these, secreted aspartyl proteases (Sap) have been the most extensively studied. It was reported that *C. glabrata* was not able to produce Sap (Ashour *et al.,* 2015), and very recently, *C. bracarensis* was shown to secrete very low levels of Sap (Moreira *et al.,* 2015).

C. bracarensis and *C. glabrata* strains were cultured for 11 days in YCB-BSA plates at 30°C followed by staining with amido black that binds and stains proteins with a dark blue color. As shown in Figure 4, only *C. albicans* (A) was surrounded by a halo without staining, corresponding to degraded BSA, caused by the production of Saps. The remaining strains showed no halo around themselves, which indicates lack of Sap production. Cultures incubated at 37°C were equally stained, and the obtained result was analogous to that shown in Figure 4.

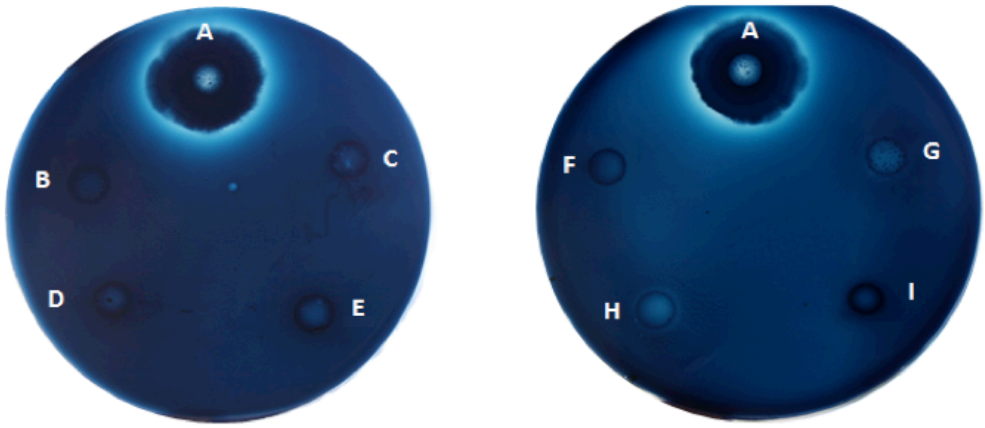


Figure 4: **Evaluation of Sap production by *C. glabrata* and *C. bracarensis* strains in YCB-BSA medium stained with amido black.** Images were captured after 11 days of growth at 30°C. *C. albicans* was used as a positive control. **A**- *C. albicans* SC5314, **B**- *C. glabrata* hemo117, **C**- *C. bracarensis* CL-7030, **D**- *C. bracarensis* 445L, **E**- *C. bracarensis* NCYC 3133, **F-** *C. glabrata* CBS 138, **G**- *C. bracarensis* 246188, **H-** *C. glabrata* CIP088, **I**- *C. bracarensis* 153M.

1.3 Filamentous grown

Virulence factors such as filamentation contribute to *Candida* pathogenesis and hyphae appear to be more invasive than the yeast form in several species of the genus *Candida* (Brunke and Hube, 2013). For this reason we tested all *C. glabrata* and *C. bracarensis* strains in what concerns filamentation ability. As described in literature (Mayer *et al.,* 2013; Si *et al.,* 2013) *C. albicans* was able to filament in all media tested. On the opposite, *C. glabrata* and *C. bracarensis* strains did not filament in any of these culture conditions (Figure 5).

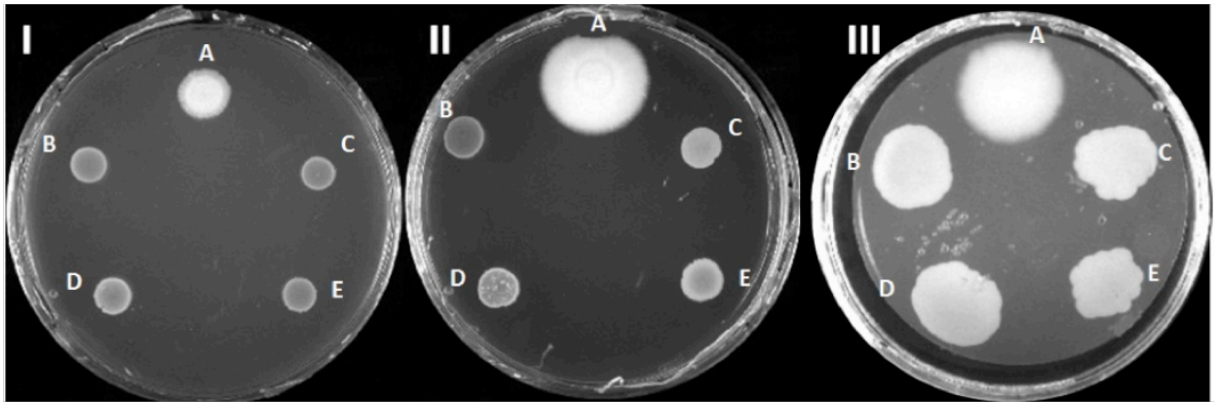


Figure 5: *Candida* strains cultured in different filamentation induction media. Images were captured after 10 days of growth at 37°C in **I-** SLAD medium**, II-** Spider medium**, III-** YPD+10%FBS medium**. A-** *C. albicans* SC5314, **B-** *C. bracarensis* CL-7030, **C-** *C. bracarensis* 445L, **D-** ***C****. glabrata* hemo1117, **E-** *C. glabrata* CBS 138.

1.4 Resistance to oxidative, osmotic, and cell wall stress agents

In order to evaluate the response of *C. bracarensis*, *C. glabrata*, and *S. cerevisiae* to different environmental conditions and stressors, each strain was cultured in medium supplemented with several stress agents, such as cell wall stress agents SDS, caffeine, calcofluor white (CFW), caspofungin, and congo red; the osmotic stress agent NaCl; and the oxidative stress agent hydrogen peroxide (H_2O_2). Three strains of each species were tested generally displaying susceptibility patterns similar to those shown in Figure 6. Only results of the highest doses tested for each stress agent are shown.

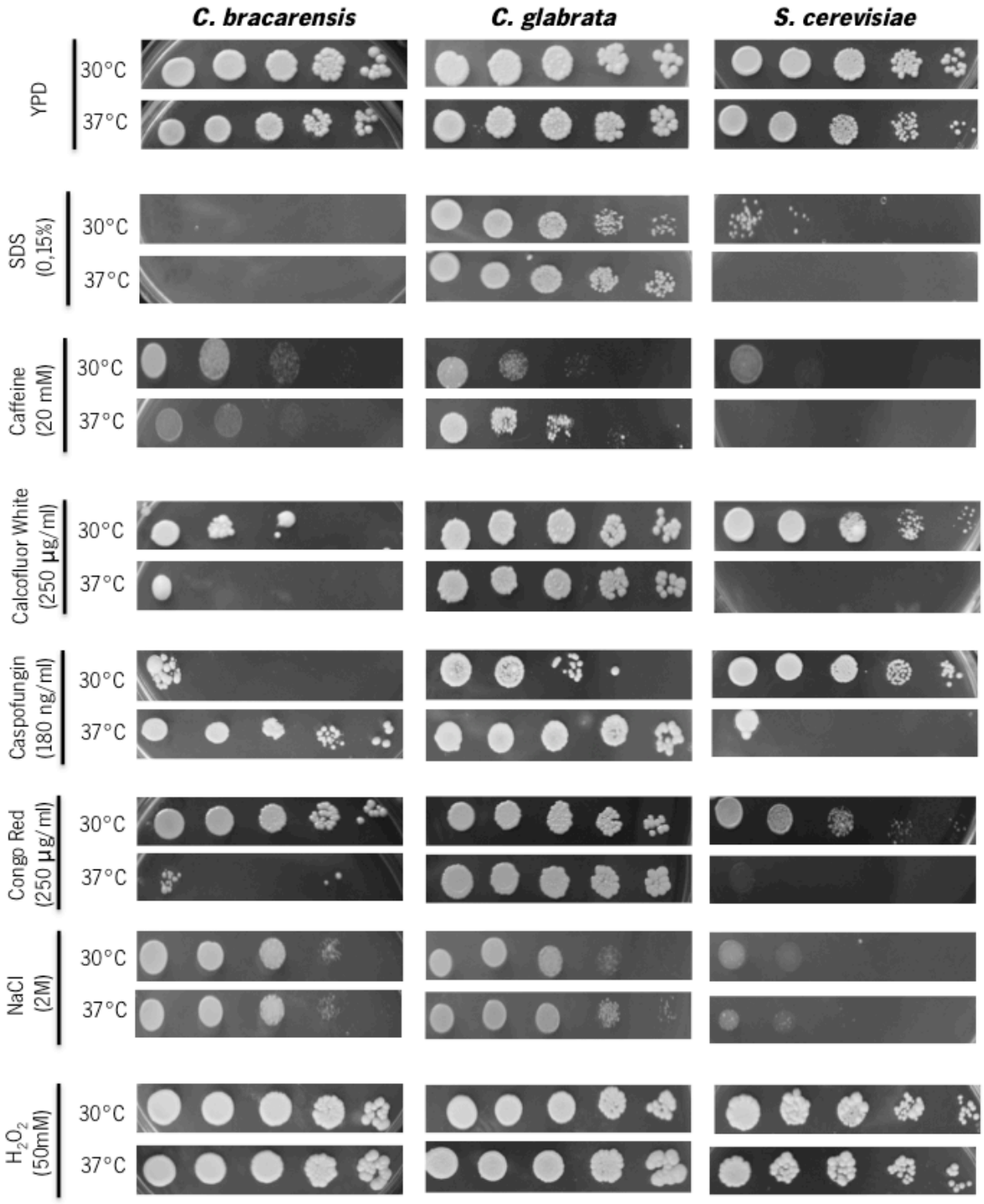


Figure 6: Growth of *C. bracarensis* 153M, *C. glabrata* CIP088 and *S. cerevisiae* L559 strains in medium supplemented with different stress agents. Serial 10-fold dilutions in YPD overnight cultures were spotted on YPD plates with: 0,15% SDS , 20 mM caffeine, 250 µg/ml calcofluor white, 180 µg/ml caspofungin, 250 µg/ml congo red or 2 M NaCl, as indicated. Susceptibility to hydrogen peroxide was accessed upon a 3 hour incubation period, of the overnight cultures, in YPD suplemented with 50 mM H_2O_2. Serial dilutions were then platted in YPD plates. All plates were incubated for 48h at 30°C or 37°C. Culture plates without any stressor were used as controls (YPD).

C. glabrata showed reduced susceptibility to SDS, both at 30°C and 37°C, an indicator of cell wall integrity (Turmachev *et al.,* 1994). On the contrary, *C. bracarensis* failed to grow in these conditions.

Caffeine is also a cell wall stress agent, and the mechanism by which it induces cell wall stress is not yet clearly understood (Levin, 2011). All species were somewhat affected by caffeine, although *C. glabrata* presented higher tolerance when cultured at 37°C in comparison to *C. bracarensis* and *S. cerevisiae*. CFW is a well characterized wall stressor agent. It binds to chitin of fungal cell walls. It is responsible for cell wall integrity disruption and entails different mechanisms of inhibition (Kingsbury et al., 2012). Hereupon, it was clear that *C. glabrata* was not affected by CFW contrary to what was observed in *C. bracarensis* and *S. cerevisiae*.

Caspofungin is one of the most used echinocandin drugs in clinical practice. Caspofungin is a lipopeptide that inhibits the activity of β–1,3–glucan synthase affecting cell wall assembly (Rueda *et al.,* 2014). All species were affected by this echinocandin at different extents. Interestingly, *C. bracarensis* and *C. glabrata* were more sensitive to caspofungin when incubated at 30°C than at 37°C. Conversely, S. cerevisiae was far more sensitive to the drug at 37°C than at 30°C. Noteworthy, although *C. glabrata* and *C. bracarensis* presented similar tolerance to caspofungin at 37°C, at 30°C however *C. bracarensis* growth in the presence of caspofungin was considerably more affected than that of *C. glabrata*.

Furthermore, *C. glabrata* was the only species which grew without limitation in medium supplemented with congo red, that despite interacting with various polysaccharides, exhibit a particularly high affinity for chitin and β-glucans (Nodet *et al.,* 1990). Susceptibility to NaCl was related with poor osmotic adaptation and reduced membrane fluidity (Khaware *et al.,* 1995). *C. bracarensis* and *C. glabrata* had similar susceptibility to NaCl 2M, however *S. cerevisiae* was considerably more susceptible to this osmotic stress agent.

Incubation with 50 mM hydrogen peroxide, in these experimental conditions, did not cause any growth limitation in *C. glabrata, C. bracarensis*, and *S. cerevisiae*.

2. Cell wall characterization

2.1 Sugar analysis and glycosidic linkage analysis

Although the interactions between cell wall glucans and immune cells have been widely investigated (Ruiz-Herrera *et al.,* 2006; Hall, 2015), how the different polysaccharides are organized and how this distribution varies from species to species is yet poorly understood. In line with that, the neutral sugar composition and glycosidic linkage of the cell wall of *C. bracarensis*, *C. glabrata*, and *S. cerevisiae* were evaluated by using GC-FID and GC-MS, respectively.

Sugar analysis (Table 2) of AIR showed that *C. bracarensis* was composed by approximately 59 molecular percentage (mol%) of glucose and 37 mol% of mannose. *C. glabrata* presented approximately 59 mol% of glucose and 38 mol% of mannose while *S. cerevisiae* was composed by approximately 54 mol% glucose and 42 mol% mannose. The remainded mol%, approximately 4 %, was related with xylose, arabinose and ribose fractions content. The results obtained of the water extraction residues revealed similar results, without significant differences among the species in study.

Contrarily to the results obtained in the sugar analysis, the glicosidic-linkage analysis (Table 3) demonstrated significant differences among species, especially between *C. bracarensis* and *C. glabrata*. *C. bracarensis* presented a higher content of β-1,3-glucan relatively to other samples studied. In contrast, the content of α -1,2-mannose was higher in *C. glabrata* and *S. cerevisiae.*

The differences found between the total molar percentage of sugar analysis and glycosidic linkage analysis were related to the structure of mannoproteins. The mannoproteins of the studied yeasts have shorter chains, and more terminal branches. For such smaller structures, the hydrolysis performed using 1M sulphuric acid is awfully drastic to mannan oligomers, leading to their degradation. The hydrolysis with TFA performed in glycosidic linkage analysis is less aggressive minimizing, on the one hand, the degradation of smaller polymers and ensuring, on the other hand, the hydrolysis of larger polymers.

Table 2: Neutral sugar analysis of *C. bracarensis*, *C. glabrata* and *S. cerevisiae*: Yeast carbohydrate composition of yeast fractions obtained by AIR and 100°C water extraction. Mean results are presented in mass concentration and molecular concentration. Each condition was set in triplicate.
Rib- Ribose, Ara-Arabinose, Xyl- Xylose, Man-Mannan, Glc- Glucose.

	Carbohydrates Concentration (mg/g)						Carbohydrates (Mol %)				
	Rib	**Ara**	**Xyl**	**Man**	**Glc**	**Total**	**Rib**	**Ara**	**Xyl**	**Man**	**Glc**
***C. bracarensis* 153M**											
AIR	13	2	1	185	298	499	3	1	0	37	59
Residue H_2O 100° C	6	8	14	101	217	345	2	2	4	29	62
***C. glabrata* CBS 138**											
AIR	17	2	1	179	255	455	5	1	0	39	56
Residue H_2O 100 ° C	14	1	0	107	199	321	5	0	0	33	61
***C. glabrata* hemo1117**											
AIR	10	2	0	178	297	488	2	1	0	36	61
Residue H_2O 100 ° C	4	0	0	105	171	281	2	0	0	37	61
***S. cerevisiae* L557**											
AIR	11	1	1	195	264	472	3	0	0	41	56
Residue H_2O 100 ° C	6	1	0	113	217	337	2	0	0	33	64
***S. cerevisiae* L560**											
AIR	8	1	2	109	129	248	4	0	1	43	51
Residue H_2O 100 ° C	-	-	-	-	-	-	-	-	-	-	-

Table 3: Yeast glycosidic linkage composition (molecular %) of sequencial extraction (AIR and 100°C water).

The glycosidic-linkage composition was determined by GC-MS of partially methylated alditol acetates. Xyl- Xylose; Man- Mannan; Glc- Glucose

	***C. bracarensis* 153M**		***C. glabrata* CBS 138**		***C. glabrata* hemo1117**		***S. cerevisiae* L557**		***S. cerevisiae* L560**	
	AIR	Sn H_2O 100°C	AIR	Sn H_2O 100°C	AIR	Sn H_2O 100°C	AIR	Sn H_2O 100°C	AIR	Sn H_2O 100°C
4-Xyl	-	-	-	-	-	1,6	-	-	-	-
Total	**-**	**-**	**-**	**-**	**-**	**1,6**	**-**	**-**	**-**	**-**
t-Man	23,3	27,4	24,8	34,9	21,7	20,0	23,7	27,1	21,6	-
2-Man	3,8	5,9	19,3	26,1	18,4	16,5	13,7	17,4	13,7	-
4-Man		1,2	-	-		16,5		-		-
6-Man	1,9	2,5	2,4	1,4	2,5	2,4	2,2	3,9	2,0	-
2,6-Man	15,9	24,0	16,4	13,4	15,1	13,0	20,7	23,2	17,9	-
3,6-Man		1,2		-	-	-		-		-
2,3,4,6-Man	0,3	1,0	2,1	0,9	1,9	2,4	0,5	1,3	1,4	-
Total	**45,0**	**63,1**	**64,9**	**76,7**	**59,6**	**70,9**	**60,8**	**73,0**	**56,7**	-
t-Glc	7,6	3,8	4,3	1,8	5,1	2,8	4,8	1,4	3,2	-
3-Glc	19,2	10,1	6,0	0,9	4,0	1,1	7,3	0,8	8,1	-
4-Glc	16,7	17,0	13,4	18,6	19,3	27,4	8,6	9,8	5,2	-
6-Glc	5,8	2,3	5,4	0,6	5,3	1,2	11,7	1,5	5,6	-
2,3-Glc	1,7	0,6	0,4	-	1,0	-	1,3	-	1,2	-
2,4-Glc	-	-	-	-	0,3	-	-	-		-
4,6-Glc	0,9	0,8	-	-	2,1	2,3	-	-		-
3,6-Glc	1,7	0,5	0,6	-	0,9	-	-	-		-
2,3,4,6-Glc	1,3	1,8	4,9	3,2	2,4	9,2	5,5	13,5	20,0	-
Total	**55,0**	**36,9**	**35,1**	**25,1**	**40,4**	**43,9**	**39,2**	**27,0**	**43,3**	**-**

2.2 Determination of β -1,3 glucan exposure

The exposition of β-1,3-glucans on the cell wall surface of *C. bracarensis, C. glabrata*, and *S. cerevisiae* was evaluated by flow cytometry upon incubation with an anti-β-1,3-glucan monoclonal antibody (mAb). β-1,3-glucans are usually localized in the inner layer of the cell wall and limitedly accessed (Brown *et al.,* 2014). To unmask this polysaccharide and increase antibody recognition, cells were HK at 70°C for 30 minutes.

As can be observed in Figure 7, the mean fluorescence intensity (MFI), due to β-1,3-glucan staining, is markedly higher in *C. bracarensis* than in the other species indicating that higher antibody recognized epitopes were available in the former species.

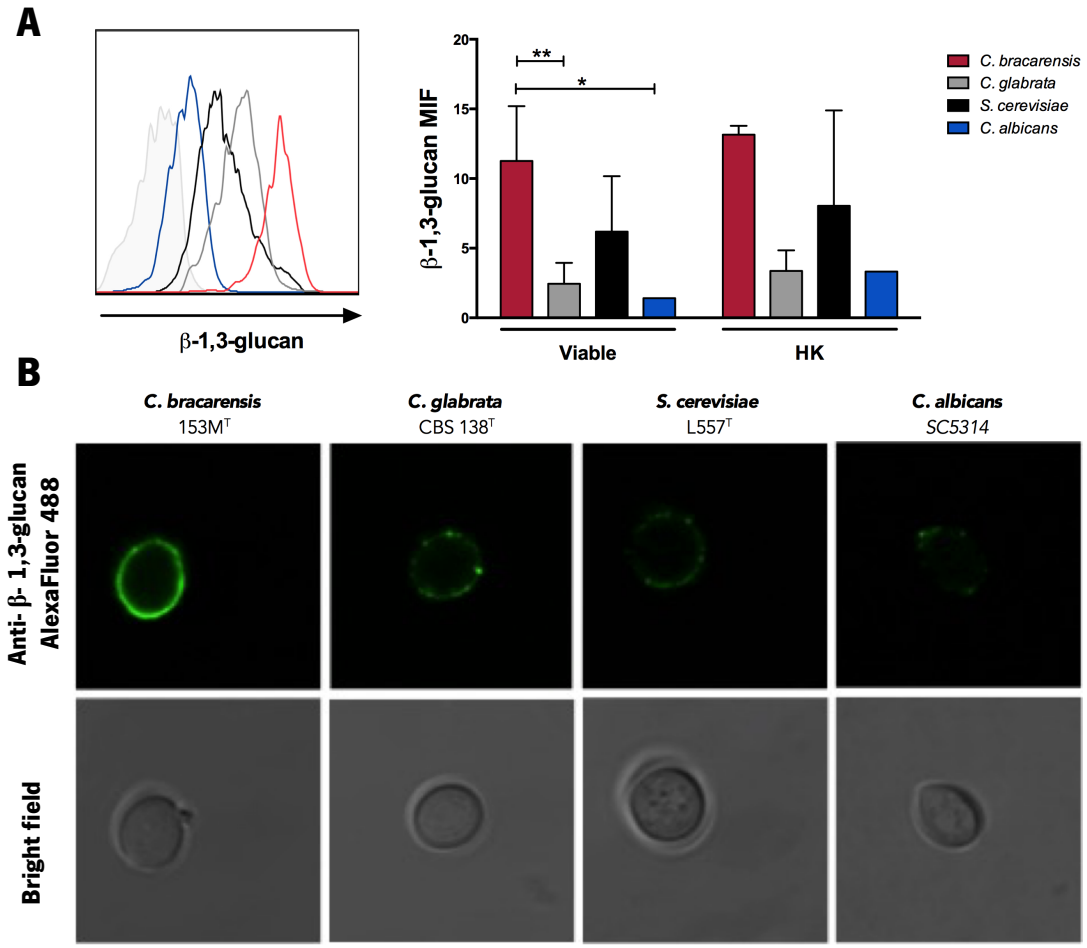


Figure 7: Determination of β-1,3 glucan exposed content in *C. bracarensis, C. glabrata, S. cerevisiae* and *C. albicans* cell wall. Yeasts were incubated with an anti-β-1,3-glucan mAb , revealed with an anti-mouse IgG conjugated with Alexa Fluor 488, and analysed by flow cytometry (A), and confocal microscopy (B). Bars correspond to means plus one SD. One-way ANOVA with Bonferroni post-Hoc test. *C. bracarensis* n=3; *C. glabrata* n=3; *S. cerevisiae* n=3 and *C. albicans* n=1 (*P<0,05; ** P<0,01).

Heat-killed yeasts had slightly increased β-1,3-glucan exposure in all species, maintaining the same trend observed for viable yeasts. In accordance, *C. bracarensis* yeasts showed a significantly brighter signal than *C. glabrata*, *S. cerevisiae*, and also *C. albicans*, when stained with anti-β-1,3-glucan mAb, and analyzed by confocal microscopy (Figure 7).

2.3 Determination of yeast cell wall porosity: Polycations assay

The ability of the cell wall to protect the yeast by limiting access to outside molecules also provides a potential barrier to diffusion of fungal products (Harris *et al.*, 2009; Ene *et al.,* 2012). Given the major impact on cell wall architecture, the wall porosity was investigated using an assay based on the polycation-induced leakage of UV-absorbing compounds from cells (Table 4). This assay compares leakage induced by small polycations (poly-L-lysine, 50 KDa) with the release caused by large polycations, such as DEAE-dextran (500 KDa). Disturbance of the cell membrane by DEAE-dextran highly depends on the degree of porosity of the cell wall (De Nobel *et al.,* 1990).

Table 4: Effect of polycations on *C. bracarensis*, *C. glabrata*, *S. cerevisiae*, and *C. albicans* cell membrane. Absorbance values with DEAE-Dextran and Poly-L-lysine treatment and percentage of relative cell wall porosity ± SD. One-way ANOVA with Bonferroni post-Hoc test. *C. bracarensis* n=3; *C. glabrata* n=3; *S. cerevisiae* n=3 and *C. albicans* n=1 (**** P<0.0001) *C. bracarensis* vs other yeast species.

Yeast	Cell leakage (A_{260nm})		Relative porosity (%)
	DEAE-dextran 500 KDa	Poly L-Lysine 50 KDa	
C. bracarensis	0,121	0,138	68,9 ± 3,1****
C. glabrata	0,013	0,116	2,2 ± 0,8
S. cerevisiae	0,019	0,056	3,2 ± 0,4
C. albicans	0,037	0,070	4,6 ± 3,1

The cell wall relative porosity of *C. bracarensis* was more than tenfold higher than that of *C. glabrata* and *S. cerevisiae*. Increased UV-absorving compound release to the extracellular environment was induced by DEAE-dextran in *C. bracarensis*, when compared with any of the other species. Increased porosity has been previously associated with short mannan side-chains (Ene *et al.,* 2012). Thus, these values of *C. bracarensis* cell wall porosity are in accordance with the results described above related to the cell wall composition.

3. Quantification of yeasts phagocytosis by macrophages

Phagocytosis plays a critical role in innate immunity, facilitating the removal and killing of pathogens, and priming the adaptive immune response (Rubin-Bejerano, 2007; Romani, 2011). The phagocytic process is initiated by the cross-linking of an array of surface receptors, some capable of direct recognition and others that recognize opsonins coating the pathogen (Herre *et al.,* 2004).

Phagocytosis assays were first performed in RAW 246.7 macrophages incubated with heat-killed (HK) yeasts and *C. glabrata* was phagocytosed at higher rates than *C. bracarensis* and *S. cerevisiae*. *S. cerevisiae* was the least phagocytosed species (Figure 8 B). To evaluate if the method used to inactivate yeasts could influence their recognition by macrophages and thus impact on the phagocytosis rate, fixed yeasts were incubated with RAW 246.7 macrophages and regardless of the method used to inactivate the cells, *C. glabrata* was still the most phagocytosed species while *S. cerevisiae* was the least phagocytosed (Figure 8 C). Since sugar cell wall composition was performed in yeast cells grown in Sabouraud medium, the assay referred above for HK yeast cells was also performed in cells grown in Sabouraud medium to confirm that growth in Sabouraud did not affect yeast phagocytosis by macrophages. The results obtained were similar to those seen with yeast cells grown in YPD, thus confirming that growth in the absence of yeast extract did not greatly affect phagocytosis (Figure 8 D). The phagocytosis rates of the different yeast species were also determined in human monocyte-derived macrophages. Similarly to what had been observed with RAW 246.7 macrophages, *C. glabrata* cells were engulfed at a greater extent by MDMs and once again *S. cerevisiae* was the least internalized species (Figure 8 E).

To evaluate if viable yeast cells could interfere with the phagocytosis process, phagocytosis assays were performed using viable yeast cells and the results were analyzed by flow cytometry (Figure 9 A) and CFU counts (Figure 9 B). *C. glabrata* remained the most phagocytosed species and *S. cerevisiae* was internalized at lower rates than the other species. However, the statistical significance of the differences in the phagocytosis rates between *C. glabrata* and *C. bracarensis* found when using HK or fixed yeast cells were lost.

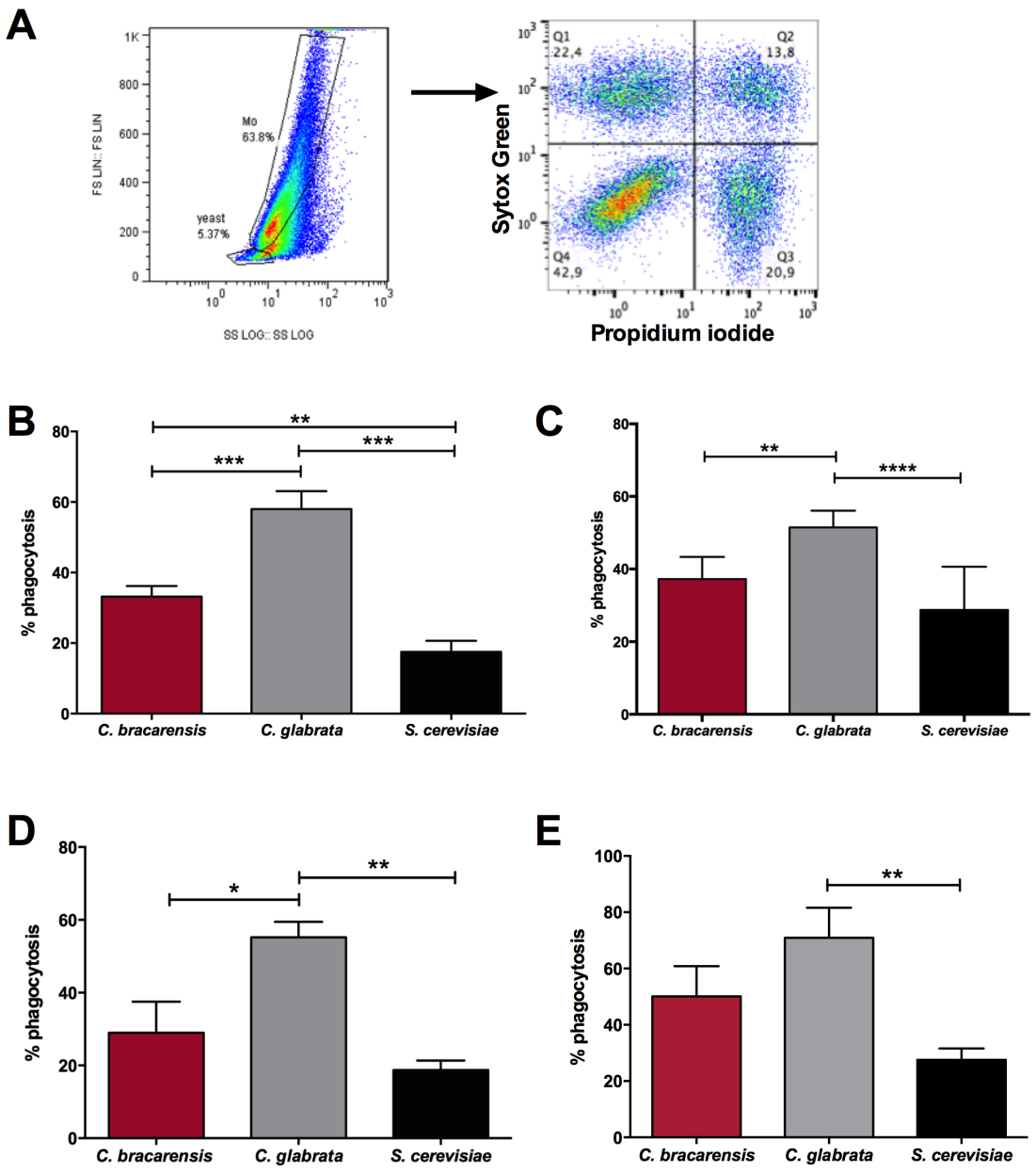


Figure 8: Phagocytosis of killed *C. bracarensis, C. glabrata* and *S. cerevisiae* Quantification of yeast phagocytosis by macrophages was assessed by flow cytometry. Macrophages (MΦ) were incubated for 30 min with inactivated yeasts labelled with Sytox Green and stained with Propidium Iodide prior to analysis (A) Representative FACS examples. After gating the macrophage population (left) the quantification of four sub-populations was performed (right) Q1: MΦ only with internalized yeasts; Q2: MΦ with internalized and adhered yeasts; Q3: MΦ only with adhered yeasts; Q4: MΦ without interaction with yeasts. RAW 246.7 murine macrophage cell line phagocytosis percentage of (B) HK yeasts grown in YPD medium, (C) fixed (formol-ethanol) yeasts grown in YPD media and (D) HK yeasts grown in Sabouraud medium. Phagocytosis assays of HK yeasts, grown in YPD medium, were also performed with human monocyte-derived macrophages (E). Each condition was set in triplicate. Bars represent means plus one SD. One-way ANOVA with Bonferroni post-Hoc test: n=5 per yeast species in RAW 246.7 phagocytosis assays and n=3 per yeast species in human monocyte-derived macrophage assays (*P<0,05; ** P<0,01; *** P<0,001; ****P<0,0001).

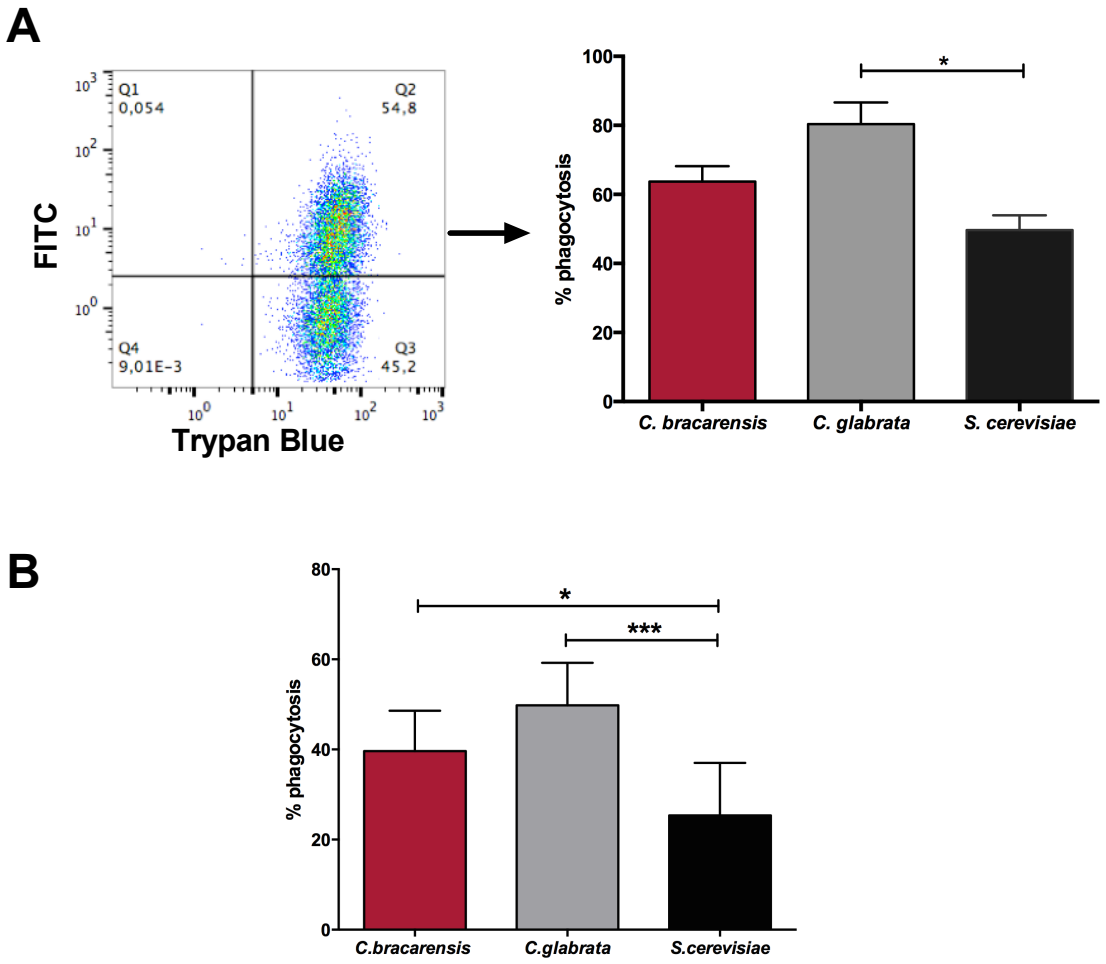


Figure 9: Phagocytosis of viable *C. bracarensis, C. glabrata* and *S. cerevisiae* Quantification of yeast phagocytosis by RAW 246.7 macrophage cell line was assessed by flow cytometry (A) and by CFU counting (B). For FACS analysis MΦ were incubated during 30 min with viable yeasts labelled with FITC and stained with Trypan Blue right before analysis. Representative FACS examples are shown on the left. Q2: MΦ with internalized yeasts; Q3: MΦ without interaction with yeasts. Alternatively, unstained live yeasts uptake was assessed by CFU counts after plating of the supernatants obtained by extensive washing of the yeast/macrophage cultures on YPD plates. Each condition was set in triplicate. Bars represent means plus one SD. One-way ANOVA with Bonferroni post-Hoc *C. bracarensis* n=2; *C. glabrata* n=2; *S. cerevisiae* n=2 (*P<0,05; *** P<0,001).

Taking into account the different experimental conditions tested, *C. glabrata* was generally more efficiently internalized by macrophages than its closely related species *C. bracarensis* and *S. cerevisiae*

Given that *C. glabrata*, *C. bracarensis*, and *S. cerevisiae* had different cell wall composition, distinct exposure of β-1,3-glucans, and unique susceptibility patterns to several cell wall stress agents, here we tried to block recognition/signalling by specific host phagocytic receptors that could be differentially interacting and internalizing yeasts. Thus, murine macrophages (RAW 246.7 cell line

and resident peritoneal macrophages) were incubated with soluble mannan or laminarin (a β-1,3-glucan) prior to culture with viable yeast cells to block the mannose receptor and dectin-1 receptor, respectively. Addicionally, specific Dectin-1 blockage was done by treating macrophages with an anti-mouse Dectin-1 mAb before adding the yeast cells. RAW macrophages were also treated with anti-mouse/human CR3/CD11b in order to block CR3 receptor. Incubation with soluble mannan and anti-CR3/CD11b did not affect significantly the phagocytosis rates of any yeast species by RAW 246.7 macrophages (Figure 10 A). In this macrophage cell line, blockage of dectin-1 using laminarin did not affect the uptake rates of *C. bracarensis*, but clearly reduced the phagocytosis rates of the other yeast species by RAW 246.7 macrophages, particularly of *S. cerevisiae* (Figure 10 A). Blockage of the Dectin-1 receptor with anti-mouse Dectin-1 mAb confirmed in general the results obtained by dectin-1 blockage with laminarin, nevertheless, uptake of *C. bracarensis* was reduced upon blocking mAb treatment (Figure 10 A). Curiously, *S. cerevisiae* was phagocytosed at higher rates by RPMs than *C. bracarensis*. Even so, *C. glabrata* remained the most internalized species (Figure 10 B). In general, the effect of laminarin treatment on RPMs phagocytosis was similar to the one observed on RAW 246.7 macrophages. Intringuingly, when RPMs were pre-treated with anti-mouse Dectin-1 mAb, no changes in the phagocytosis rates could be observed for any of the yeast species tested (Figure 10 B).

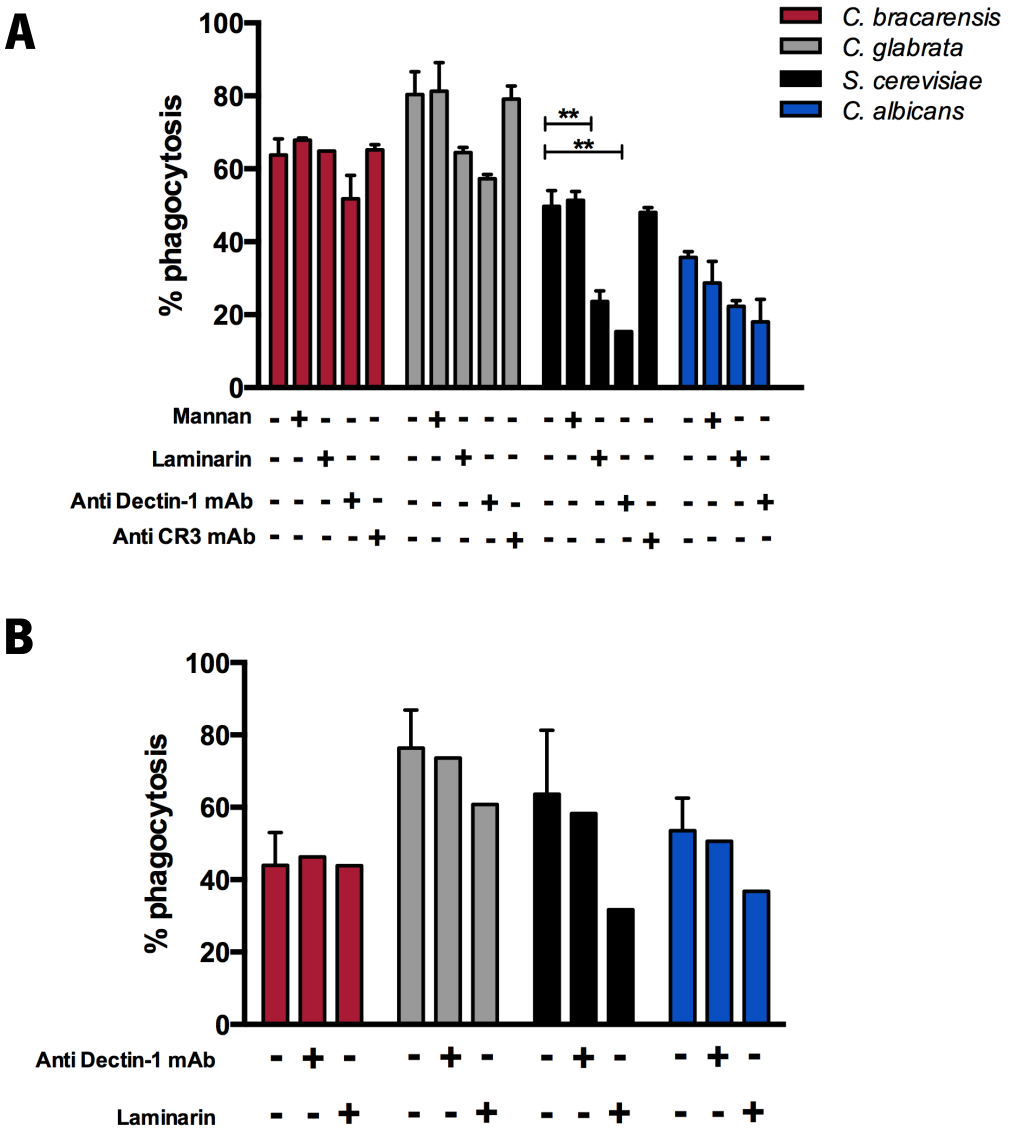


Figure 10: Phagocytosis of viable *C. bracarensis, C. glabrata* and *S. cerevisiae* with or without PRRs signaling inhibitors. Quantification of yeast phagocytosis by RAW 246.7 macrophage cell line and murine resident peritoneal macrophages was assessed by flow cytometry For FACS analysis MΦ were incubated during 30 min with viable yeasts labelled with FITC and stained with TB right before analysis. RAW 246.7 were incubated with soluble mannan (500 μg/ml), laminarin (500 μg/ml), anti-mouse Dectin-1 mAb (3 μg/ml) and with anti-mouse/human CD11b/CR3 (20 μg/ml) 30 min prior infection with yeasts (A). Inhibition of murine resident peritoneal macrophages by Laminarin or Anti-Dectin-1 mAb (B) was similarly performed. Bars represent means plus one SD. Unpaired two-tailed t-test *C. bracarensis* n=2; *C. glabrata* n=2; *S. cerevisiae* n=2 (** P<0,01).

4. Determination of Reactive Oxygen Species production by macrophages

Phagocytes play a crucial role in the host response to fungi and the production of reactive oxygen metabolites is a key event following phagocytosis (Nathan and Shiloh, 2000; Seider *et al.,* 2014 being considered a major antifungal mechanism in phagocytes (Cheng *et al.,* 2012). Both *C. albicans* and *C. glabrata* produce enzymes and molecules to evade oxidative killing, which may represent an important immune evasion mechanism (Seider *et al.,* 2014). For that, we examined the ability of *C. bracarensis*, *C. glabrata* and *S. cerevisiae* to induce and modulate phagocyte ROS production.

As presented in Figure 11 A, *C. glabrata* and *C. bracarensis* stimulated similar ROS production by RAW 246.7 macrophages. On the other hand, *S. cerevisiae* induced the lowest levels of ROS.

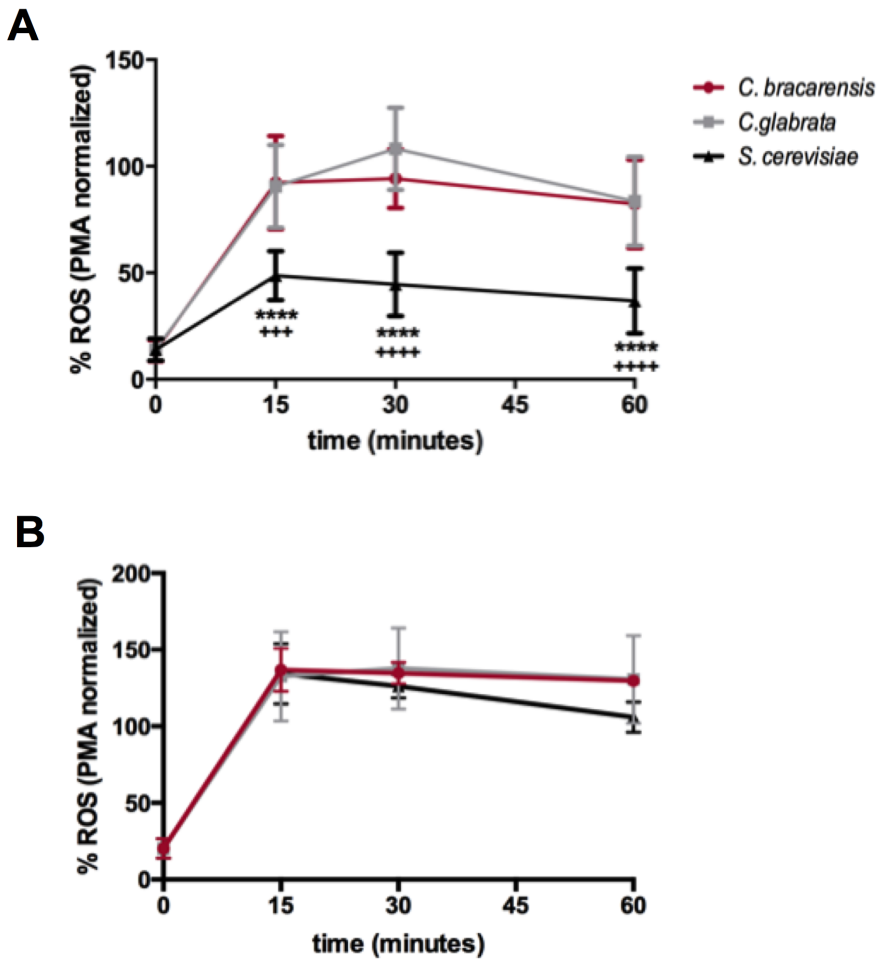


Figure 11: Production of ROS by murine macrophages ROS quantification was assessed by using a Superoxide detection Kit. Macrophages were incubated for 15, 30 and 60 min with live (A) or HK (B) yeasts and analysed by flow cytometry. Results were normalized to macrophages stimulated with PMA (positive control). Each condition was set in triplicate. Means ± one SD are presented. One-way ANOVA with Bonferroni post Hoc test *C. bracarensis* n=5; *C. glabrata* n=5*; S. cerevisiae* n=5 (+++/***P< 0,001; ++++/**** P<0,0001) * *S. cerevisiae vs. C. bracarensis*; + *S. cerevisiae vs. C. glabrata.*

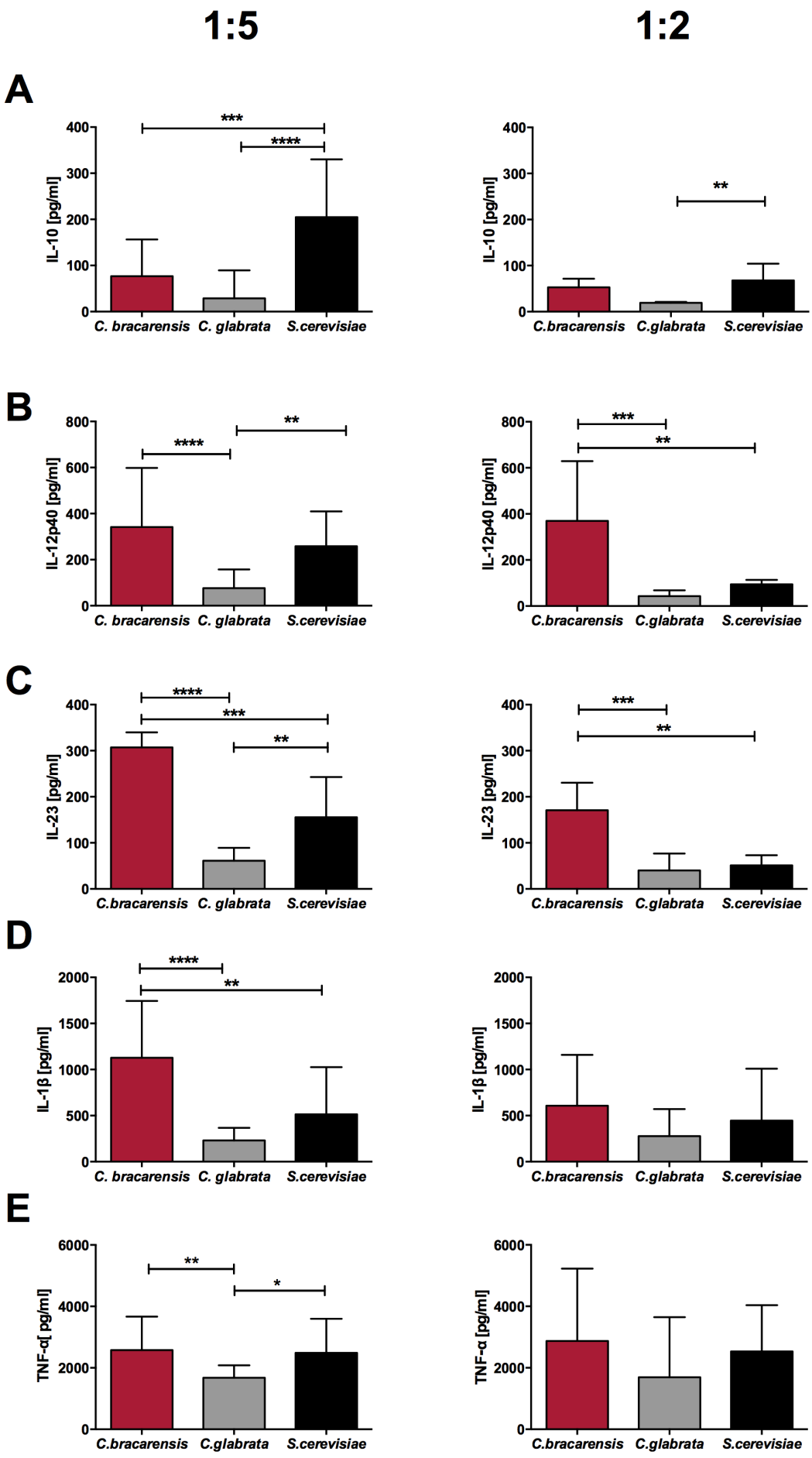
Interestingly, when this assay was performed with heat-killed yeasts, the three species stimulated equivalent ROS production and higher percentages of macrophages were ROS positive when compared with macrophages incubated with viable yeasts (Figure 11 B).

5. Quantification of cytokine production by sandwich ELISA

Following the recognition of pathogens by PRR, distinct signalling pathways are induced. These pathways act through a cross-regulation mechanism, which results in the production of proinflammatory cytokines (Mogensen, 2009).

The induction of proinflammatory cytokines is an important component of anti-*Candida* host defence (Ifrim *et al.,* 2014). Thus, the levels of the cytokines IL-12, TNF-α, IL-1β, IL-23, and also of the anti-inflammatory cytokine IL-10 were quantified in the culture supernatants of BMDCs and BMMs from BALB/c (Figure 12) and C57BL/6 (Figure 13) mice stimulated with *C. bracarensis*, *C. glabrata* and *S. cerevisiae* at two different multiplicities of infection. Supernatants from BMDCs incubated with medium alone or with LPS were used as negative and positive controls, respectively, though the results were omitted in the graphs for simplicity.

C. bracarensis induced higher production of proinflammatory cytokines such as IL-12p40, IL-23, IL-1β, and TNF-α, by BMDCs of BALB/c mice regardless of the multiplicity of infection. Globally, *C. glabrata* was the species that induced the lowest levels of the proinflammatory cytokines referred above. Curiously, *S. cerevisiae* was the species that stimulated the highest levels of IL-10. *C. glabrata* induced the lowest levels of this anti-inflammatory cytokine by BMDCs (Figure 12).


1:5
1:2
A
IL-10 [pg/ml]
400
300
200
100
0
***
****
**
C. bracarensis
C. glabrata
S.cerevisiae
C.bracarensis
C.glabrata
S.cerevisiae
B
IL-12p40 [pg/ml]
800
600
400
200
0
****
**
***
C. bracarensis
C. glabrata
S.cerevisiae
C.bracarensis
C.glabrata
S.cerevisiae
C
IL-23 [pg/ml]
400
300
200
100
0
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***
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C.bracarensis
C. glabrata
S.cerevisiae
C.bracarensis
C.glabrata
S.cerevisiae
D
IL-1β [pg/ml]
2000
1500
1000
500
0
****
**
C. bracarensis
C. glabrata
S.cerevisiae
C.bracarensis
C.glabrata
S.cerevisiae
E
TNF-α[pg/ml]
6000
4000
2000
0
**
*
C.bracarensis
C.glabrata
S.cerevisiae
TNF-α [pg/ml]
C.bracarensis
C.glabrata
S.cerevisiae

Figure 12: Quantification of cytokines in the supernatants of BMDCs from BALB/c mice.
The levels of IL-10 (A), IL-12p40 (B), IL-23 (C), TNF-α (D), and IL-1β (E) secreted by BMDC from BALB/c mice were quantified by sandwich ELISA. BMDCs were incubated with viable yeast cells at a MOI of 1:5 (1 DC/Mo: 5 yeast cells) or at a MOI of 1:2, as indicated, for 24h. The supernatants were collected and the referred cytokines were quantified. Each condition was set in triplicate. Bars correspond to means plus SD. One-way ANOVA with Bonferroni post Hoc test. *C. bracarensis* n=5; *C. glabrata* n=5; *S. cerevisiae* n=5 (*P<0,05; ** P<0,01; *** P<0,001; **** P<0,0001).

The profile of cytokines produced by BMDCs from C57BL/6 mice, upon incubation with the yeasts, was in general very similar to the one found in BMDCs from BALB/c mice. As presented in Figure 13, *C. bracarensis* stimulated the production of the highest concentrations of the proinflammatory cytokines Il-23, IL-1β and TNF-α. The Th1 driving cytokine 12p40 levels were also found increased in BMDCs incubated with *C. bracarensis*, despite not being statistically significant. Additionally, no significant differences in the levels of IL-10 were found among the supernatants of BMDCs incubated with any of the studied yeast species (Figure 13).

Overall, despite small differences between mice strains, the profile of cytokines was quite concordant. *C. bracarensis* induced the highest concentration levels of proinflammatory cytokines, with particular emphasis on IL-23 and TNF- α.

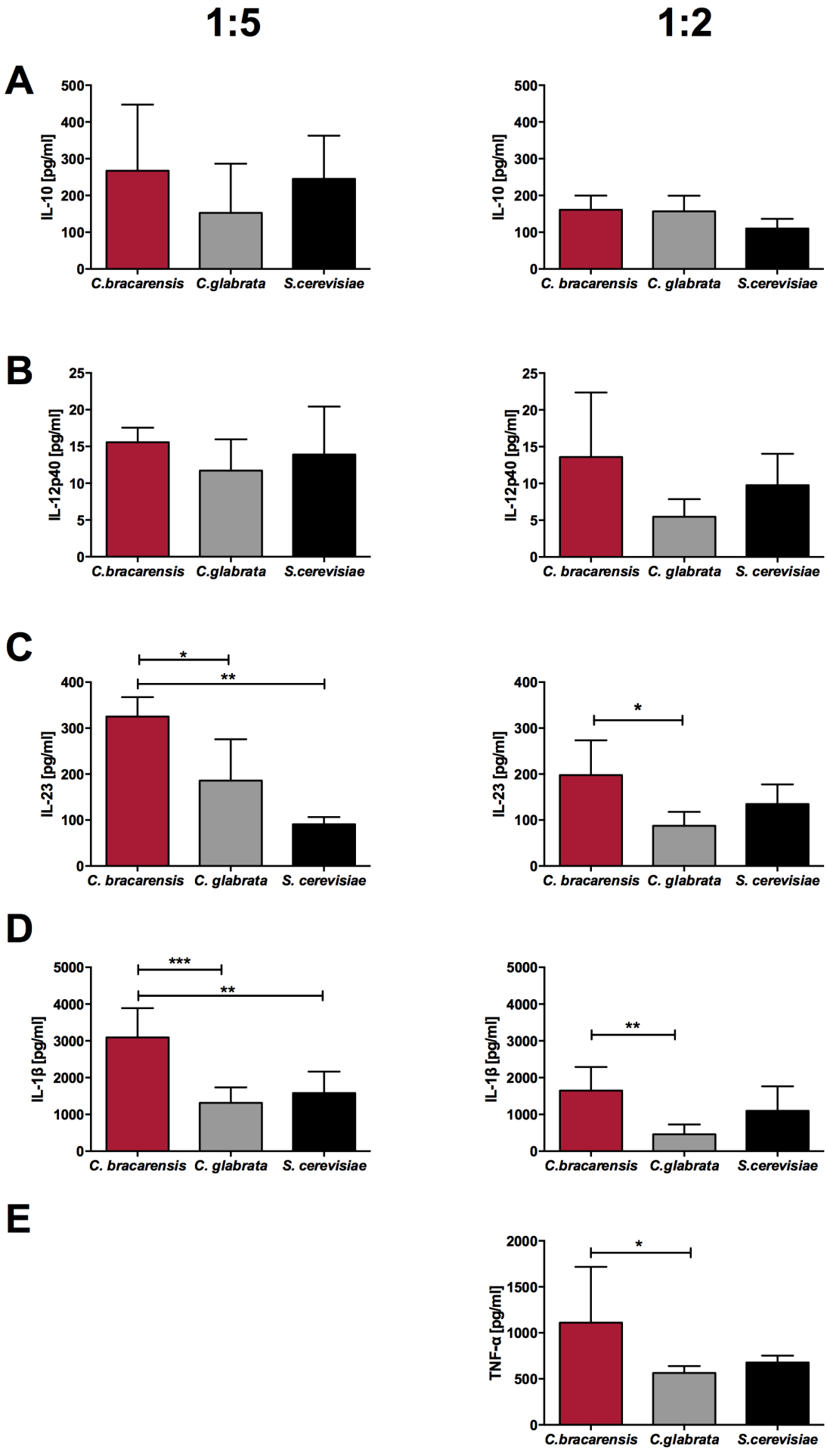
1:5
1:2
A
IL-10 [pg/ml]
500
400
300
200
100
0
C.bracarensis
C.glabrata
S.cerevisiae
C. bracarensis
C. glabrata
S.cerevisiae
B
IL-12p40 [pg/ml]
25
20
15
10
5
0
C.bracarensis
C.glabrata
S.cerevisiae
C. bracarensis
C. glabrata
S. cerevisiae
C
IL-23 [pg/ml]
400
300
200
100
0
*
**
C. bracarensis
C. glabrata
S. cerevisiae
D
IL-1β [pg/ml]
5000
4000
3000
2000
1000
0
***
**
C. bracarensis
C. glabrata
S. cerevisiae
C.bracarensis
C.glabrata
S.cerevisiae
E
TNF-α [pg/ml]
2000
1500
1000
500
0
*
C.bracarensis
C.glabrata
S.cerevisiae

Figure 13: Quantification of cytokines in the supernatants of BMDCs from C57BL/6 mice.
The levels of IL-10 (A), IL-12p40 (B), IL-23 (C), TNF-α (D), and IL-1β (E) secreted by BMDC from BALB/c mice were quantified by sandwich ELISA. BMDCs were incubated with viable yeast cells at a MOI of 1:5 (1 DC/Mo: 5 yeast cells) or at a MOI of 1:2, as indicated, for 24h. The supernatants were collected and the referred cytokines were quantified. Each condition was set in triplicate. Bars correspond to means plus SD. One-way ANOVA with Bonferroni post Hoc test. *C. bracarensis* n=3; *C. glabrata* n=3; *S. cerevisiae* n=3 (*P<0,05; ** P<0,01; *** P<0,001).

5.1. Effect of Dectin-1 and CR3 blockage on TNF-α production

PRRs, such as TLRs and C-type lectin receptors, particularly Dectin-1, are essential determinants of host antifungal immunity. The recognition of β-glucans by Dectin-1 has been shown to mediate cell activation, cytokine production, and a variety of antifungal responses (Carvalho *et al.,* 2012).

Given that *C. bracarensis* allowed greater access to cell wall β-1,3-glucans, dectin-1 and/or CR3 could be recognizing those carbohydrates and contributing to the high levels of proinflammatory cytokines detected in BMDCs incubated with that yeast species. Thus, Dectin-1 and also CR3 blockage was attempted in BMDCs and BMM infected with the different yeast species, and TNF-α levels were determined in the supernatants of the cultures to assess the involvement of Dectin-1 and CR3 in the inflammatory response to yeasts. As expected, production of TNF-α induced on both BMDCs and BMMs by *C. bracarensis* was clearly affected by blockage of Dectin-1 receptor, irrespective of the multiplicity of infection used. Conversely, TNF-α production was not adversely affected in BMDCs or BMMs infected with *C. glabrata* or *S. cerevisiae*. Interestingly, *C. albicans*-induced TNF-α production by BMMs, but not by BMDCs, was reduced upon blockage of Dectin-1 (Figure 14).

CR3 blockage did not have a detrimental effect on *C. bracarensis*-induced TNF-α production. On the opposite, an increase in the production of TNF-α was indeed observed, not only in BMDCs and BMMs cultured with *C. bracarensis*, but also in those incubated with *C. glabrata* and *S. cerevisiae*. Curiously, CR3 blockage had a strong negative impact on TNF-α production by BMDCs and BMMs infected with *C. albicans* (Figure 14)

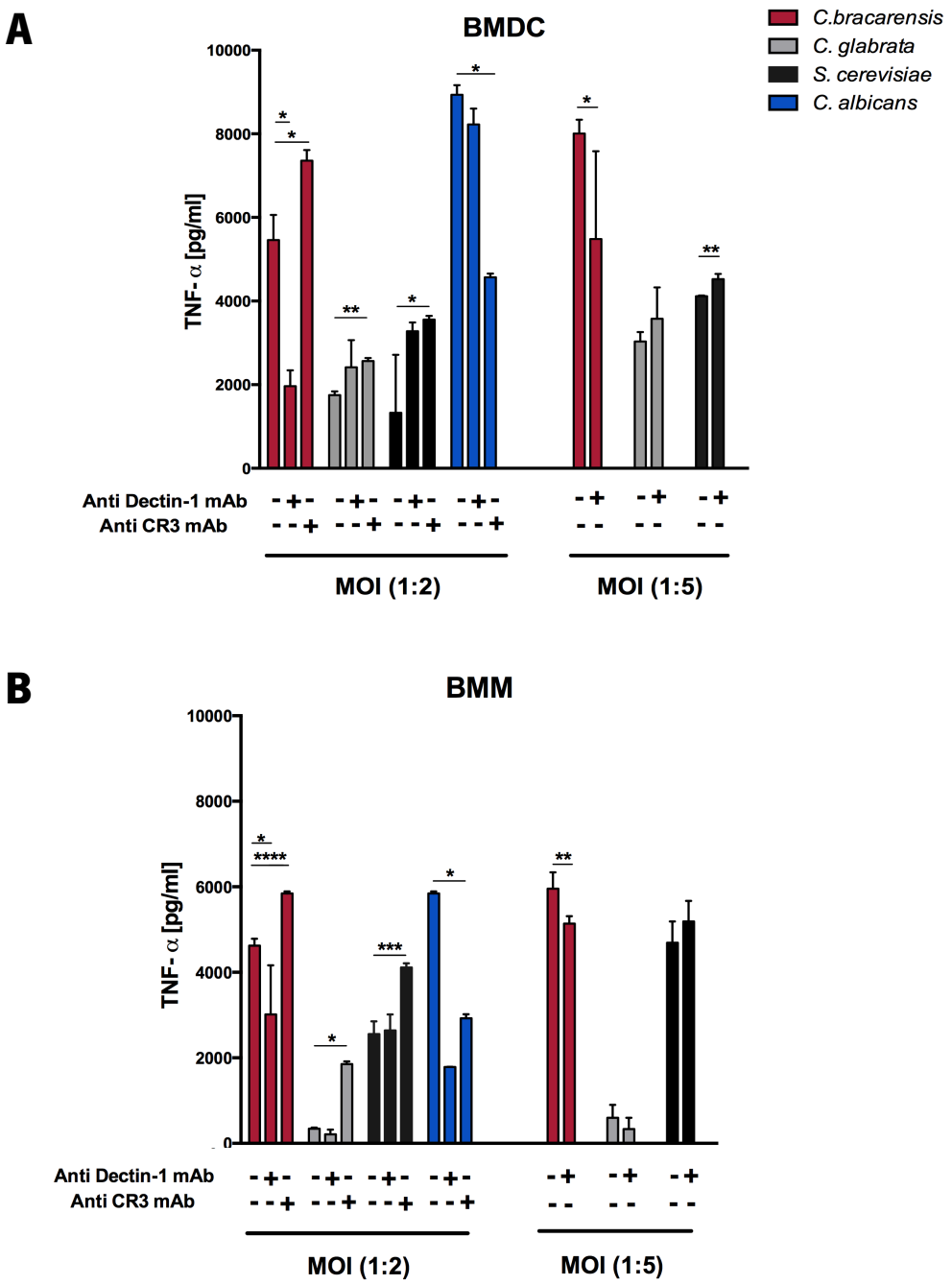

Figure 14: Quantification of TNF-α in the supernatants of BMDC and BMM from BALB/c mice without or with CR3, Dectin-1 blockage. BMDCs and BMMs were incubated with the different viable yeast strains at a MOI of 1:2 and 1:5 (1 DC/Mo: 2 and 5 yeast cells, respectively) for 24h. To block dectin-1 and CR3 signaling, anti-mouse Dectin-1 mAb (3μg/ml) and anti-mouse/human CR3 mAb (20μg/ml) were added 30 min prior to infection. The supernatants were collected and the levels of TNF-α were quantified by sandwich ELISA. Results are from a single experiment set in duplicate. Bars correspond to means plus SD. Unpaired two-tailed t-test was performed to compare, within each species, results from unblocked cultures with results from anti-Dectin-1 or anti-CR3 blocked cultures; A single strain of each yeast species was used (*P<0,05; ** P<0,01).

6. Evaluation of bone marrow derived dendritic cells activation/maturation

The role of DCs in T cell activation and in the initiation of antigen-specific immune responses is very well documented. During infection, DCs in the periphery are triggered by exposure to microbial agents or inflammatory mediators to increase their expression of major histocompatibility complex (MHC) molecules and co-stimulatory molecules such as CD80 and CD86. This activates peripheral DCs into a state ready for T cell activation (Guermonprez *et al.*, 2002; Granucci *et al.,* 2004). Activated DCs then display pathogen-encoded antigens to naive antigen-specific T cells, which respond and initiate primary T cell immune responses (Tan and O'Neill, 2005).

In order to evaluate the activation/maturation status of BMDCs after 24h of infection with yeasts, the cell surface expression of MHC class II, CD86, and CD80 activation markers was analyzed by flow cytometry upon staining with specific mAbs. All yeast species induced the maturation of BMDCs when compared with non-stimulated BMDCs (Figure 15 A). *C. bracarensis*, right after *C. albicans*, induced the highest expression of all maturation markers analyzed on BMDCs. The surface expression of these activation markers was however significantly decreased in BMDCs incubated with *C. bracarensis* upon Dectin-1 blockage (Figure 15 A and B). Blocking Dectin-1 had no marked effect on the expression of any of the markers analyzed in response to *C. glabrata* or *S. cerevisiae*. Curiously, dectin-1 blockage led to the increased expression of CD80 and CD86 on *C. albicans*-infected BMDCs (Figure 15 A),

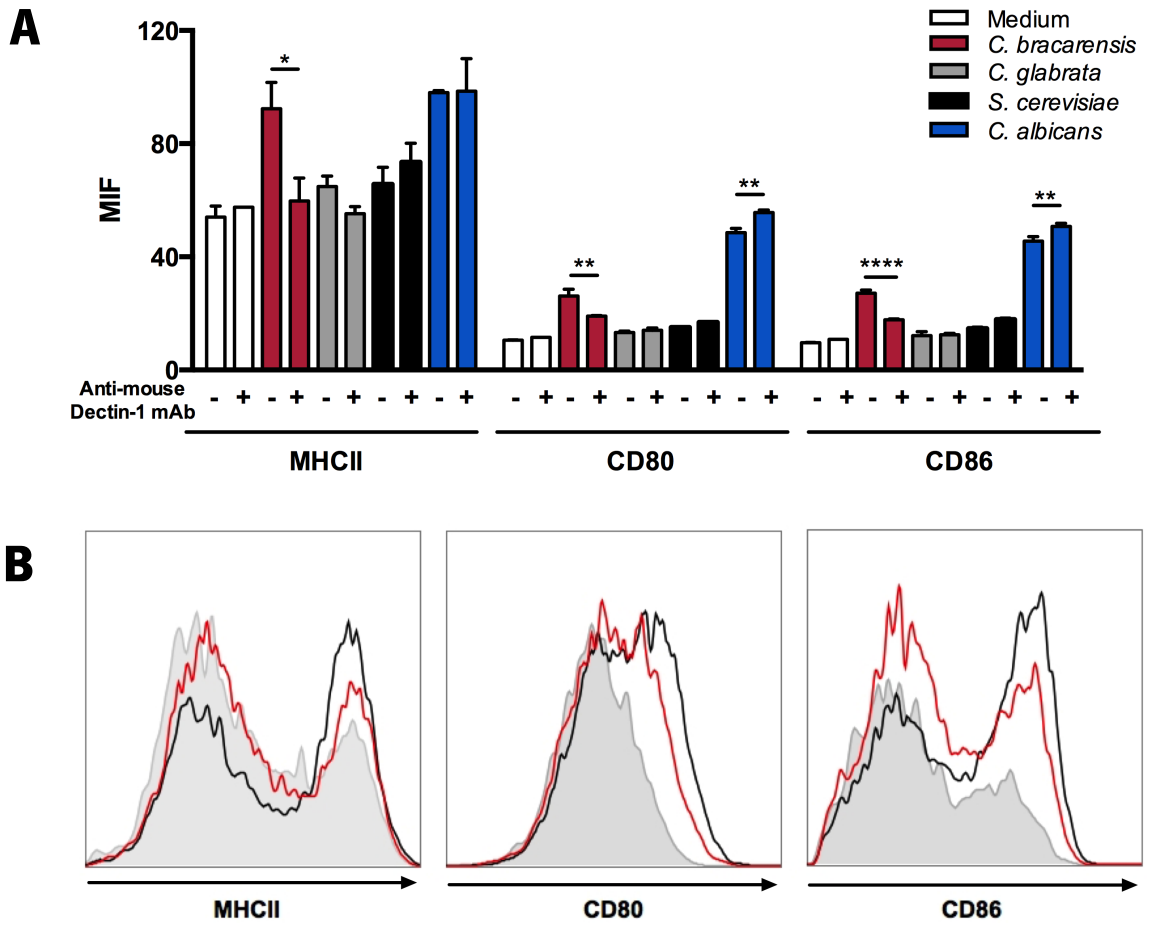

Figure 15: Yeast-induced BMDCs maturation/activation. BMDCs were incubated with viable yeast cells at a MOI of 1:2 (1 DC: 2 yeast cells) for 24h. To block dectin-1 signaling, anti-mouse Dectin-1 mAb (3µg/ml) was added 1 hour prior to infection. To analyze surface activation markers, cells were collected and blocked with FcBlock and stained with anti-CD11c-FITC conjugated, anti-MHC classII-Percp conjugated, anti-CD80-PE conjugated, and anti-CD86-PE-Cy7 conjugated, and analyzed by FACS. Results are from a single experiment set in duplicate. Bars correspond to means plus SD. B- Representative examples of histogram overlays of each activation marker analyzed on non-stimulated BMDCs (grey) and on BMDCs incubated with *C. bracarensis* without (black) or with Dectin-1 blockage (red). Bars correspond to means plus SD. Unpaired two-tailed t-test was performed to compare, within each species. *C. bracarensis* n=1 *C. glabrata* n=1; *S. cerevisiae* n=1 (*P<0,05; ** P<0,01; *** P<0,001, ****P<0,0001).

7. Early inflammatory response to *C. glabrata* and *C. bracarensis* intraperitoneal infection

Neutrophils play a crucial role in controlling and clearing fungal infection. Human polymorphonuclear neutrophils are one of the first immune cells recruited to an infection site. These cells efficiently engulf and kill microbes (Sampaio *et al.,* 2010).

The early inflammatory response to *C. bracarensis* and *C. glabrata* was evaluated in BALB/c mice 6 and 72h after intraperitoneal (i.p.) infection with 1×10^7 yeast cells. The percentage and also numbers of neutrophils, inflammatory monocytes, and macrophages recruited into the peritoneal cavity of mice infected was assessed by flow citometry using the markers CD11b, Gr-1 (Ly6 C and Ly6 G), and F4/80. Neutrophils were defined as $CD11b^{hi}Gr\text{-}1^{+}F4/80^{-}$, inflammatory monocytes were $CD11b^{+}Gr\text{-}1^{+}F4/80^{+}$, and macrophages were gated as $CD11b^{+}Gr\text{-}1^{-}F4/80^{+}$ (Taylor *et al.*, 2003).

As expected, higher recruitment of neutrophils into the peritoneal cavity was observed in both infected mouse groups, comparatively to non-infected controls, 6h after infection. However, similar percentages and numbers of neutrophils were observed in both infected mouse groups (Figure 16). Likewise, 72 h upon infection, the percentage and numbers of inflammatory monocyte in the peritoneal exudates of non-infected were significantly lower than those of infected mouse groups, while no differences could be observed between infected mouse groups (Figure 16). Conversely, the percentage and also numbers of macrophages in non-infected mice were higher than those of infected counterparts 6 h upon challenge, but 72 h after infection, although the percentage of recruited macrophages was significantly higher in the peritoneal cavity of non-infected mice, the total number of these leucocytes was lower than those of infected mice. Once again, no differences in percentage or number of macrophages in the peritoneal exudates of either *C. glabrata* or *C. bracarensis* infected mice were observed (Figure 16).

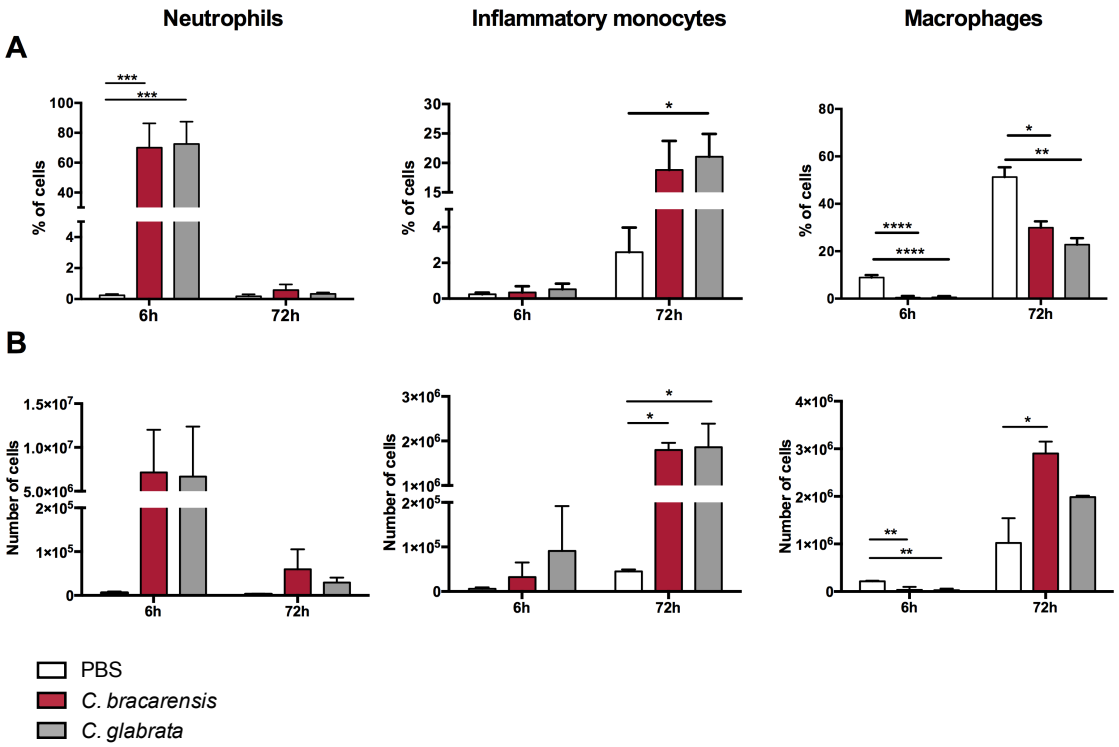

Figure 16: Early intraperitoneal inflammatory response to *C. bracarensis* and *C. glabrata*. Recruitment of neutrophils, inflammatory monocytes and macrophages into the peritoneal cavity 6 and 72 h after i.p. infection of BALB/c mice with 1×10^7 *C. bracarensis* or *C. glabrata* cells. Bars correspond to means plus SD. One-way ANOVA with Bonferroni post-Hoc test was performed to compare, within each species. PBS n=2; *C. bracarensis* n=5 (6 h; pooled data from 2 independent experiments) and n=2 (72 h); *C. glabrata* n=5 (6 h; pooled data from 2 independent experiments) and n=2 (72 h) (*P<0,05; ** P<0,01; *** P<0,001, ****P<0,0001).

Altogether these results highlight differences in cell wall composition/structure among *C. glabrata*, *C. bracarensis*, and *S. cerevisiae* that might account for the differential interaction of these yeasts with the host.

Chapter 4

General discussion and Future perspectives

C. glabrata is an important human fungal pathogen (Richardson *et al.*, 2008; Schwarzmuller *et al.*, 2014) and since 2004, when its genome was released and annotated, its comparison to *S. cerevisiae* has served to discuss possible genomic and metabolic features related to the pathogenic nature of the former species (Dujon *et al.,* 2004; Gabaldón *et al.,* 2013). Recently however, *C. bracarensis* was found to be the closest species to *C. glabrata*, and its study could be important in the clarification of infection strategies of this important pathogen.

The analysis of virulence factors has an important role in the study of pathogens and their strategies of infection. However, most of the virulence attributes of the successful pathogen *C. albicans* are absent in *C. glabrata* (Kaur *et al.*, 2005). Here, the inability of *C. glabrata* to produce Sap or hyphae was confirmed. Moreover, *C. bracarensis* strains also failed to produce Sap and to filament. However, recently it was reported that *C. bracarensis* NCYC 3133 had some proteolytic activity (Moreira *et al.,* 2015). In the present analysis, *C. bracarensis* NCYC 3133 did not have extracellular proteolytic activity, and these disparities could be associated with differences in the experimental conditions. Until now, no episodes of hypha formation in *C. glabrata and C. bracarensis* were reported, although Odds and co-workers in 1997, and also Csank and Haynes in 2000 reported pseudohypha formation in *C. glabrata*. In accordance with the results presented here, very recently, Moreira and co-workers (2015) reported the absence of filamentation in *C. bracarensis* strains cultured in RPMI medium with 10% serum. Despite the lack of some of the most important virulence characteristic of *C. albicans*, *C. glabrata* and also *C. bracarensis* seam to be capable of establishing successful infections (Rai *et al.,* 2012).

Several studies indicated that stress responses have diverged in order to generate more robust responses in *Candida*, when compared with the model yeast *Saccharomyces cerevisiae* (reviewed in Quinn and Brown, 2007). For example, fungal pathogens have evolved mechanisms to mount robust oxidative stress responses in order to resist the high levels of ROS generated by phagocytic cells. As all yeast strains used in this study grew without visible limitations in the presense of H_2O_2, it would be important to repeat this experimental assay with higher concentrations of H_2O_2 to verify if this pattern of resistance was maintained.

Also, treatment with cell wall-perturbing agents elicits in yeasts a cellular survival response known as "compensatory mechanism" that leads to an increase in the amount of β-glucan and chitin, to the production of several cell wall proteins, and to changes in the cross-linking between cell wall polymers (Arias *et al.*, 2011). Here is shown that *C. glabrata*, in opposition to *C. bracarensis* and *S. cerevisiae*, is in general more resistant to cell wall stress agents. *C. bracarensis* and *S. cerevisiae*

showed here high susceptibility to SDS, a recognized indicator of cell wall integrity (Turmachev *et al.,* 1994) due to its capacity to disrupt and solubilize the plasma membrane. On the opposite, *C. glabrata* presented almost no susceptibility to SDS. Lagorce *et al.*, 2003 suggested that increased susceptibility to SDS could be associated with a leaky cell wall surface, probably due to an increased porosity in the mannan layer. *C. bracarensis* presented a cell wall relative porosity almost 15 times higher than *C. glabrata, S. cerevisiae*, and *C. albicans*, as evaluated by using a polycation-induced leakage assay of UV-absorving compounds. This increased cell wall porosity is in accordance with the high susceptibility to SDS found in this species. Moreover, Cutler in 2001 proposed cell wall transient gaps in the mannan layer as the best hypothesis to explain yeast cell wall permeability. Indeed, it has been demonstrated that the N-linked mannans are inversely associated with cell-wall porosity in yeasts, for example, cell wall porosity decreases as the yeast cells enter stationary growth, which correlates with increasing content of N-linked mannan (De Nobel *et al.,* 1990; Cutler, 2001). However, this explanation cannot justify the susceptibility of *S. cerevisiae* to SDS, since this species had relative cell wall porosity similar to that of *C. glabrata*. Another hypothesis would be related with β-glucan cell wall content, since in the presence of SDS the content of β-1,3-glucan can be dramatically reduced, affecting the permeability and osmotic balance of the cell wall (Hong et al., 1994; Coca *et al.,* 2000). Given that *C. bracarensis* presented higher cell wall molecular percentage of β-1,3-glucans than *C. glabrata* and *S. cerevisiae*, this could also be a plausible explanation for the high susceptibility of *C. bracarensis* to SDS. Yet, this hypothesis also cannot support with the high susceptibility of S. cerevisiae to SDS, since this species had a β-1,3-glucan content similar to that of *C. glabrata*.

All yeast species tested in this study, *S. cerevisiae* in particular, demonstrated low ability to grow in presence of caffeine. *C. glabrata* was nevertheless more resistant than the other two species at 37° C. Caffeine is currently used as a phenotypic criterion to evaluate the function of the cell wall integrity pathway due to the fact that most of the mutants defective in components of cell wall integrity pathway are caffeine-sensitive (Kuranda *et al.*, 2006).

CFW and congo red are cell wall perturbing agents that bind chitin and affect its assembly within the cell wall, which results in weakening of the cell wall (Roncero and Duran, 1985; Ram et al., 2004). Aside from affecting chitin assembly, congo red also alters glucan synthesis assembly (Daher *et al.,* 2011). Once again, *C. glabrata* was the most resistant species and did not present susceptibility to these cell wall stress agents. *C. bracarensis and S. cerevisiae* were more affected by these stressors at 37° C. Curiously, hypersensitivity to CFW has been correlated with an

increase in the cell wall chitin content (He *et al.,* 2010), even though synthesis of chitin is a general compensatory mechanism to strengthen the cell wall.

Caspofungin, the most used echinochandin in clinical practice (Rueda *et al.,* 2014), interferes with cell wall biosynthesis by inhibition of β-1,3-glucan synthase (Eschenauer *et al.,* 2007; Beese-Sims *et al.,* 2012). All species demonstrated susceptibility to caspofungin, however, the highest susceptibility to caspofungin was observed in *C. bracarensis* grown at 30° C and in *S. cerevisiae* at 37°C. While the increased susceptibility of *S. cerevisiae* to caspofungin at 37°C could be attributed to the combination of a cell wall stressor and high temperature (the optimal temperature of this species is 30° C), no suitable explanation could be found for the increased susceptibility of *C. bracarensis* at 30° C. *C. glabrata* is often referred for its resistance to several antifungal drugs, namely azoles, and it was also reported that *C. glabrata* could rapidly develop resistance to echinocandins, even during short treatment periods (Perlin, 2014; Domán *et al.,* 2015). The most usual reason for caspofungin resistance is related with the chitin content, since high chitin content confers reduced susceptibility while low chitin content confers hypersensitivity (Plaine *et al*., 2008). Altogether these results suggest the existence of disparities in cell wall composition/structure among the three species. Whether *C. glabrata*, *C. bracarensis*, and *S. cerevisiae* have different chitin contents, and if and how cell wall stress agents affect this cell wall component proportion, remains to be clarified. Nevertheless, *C. glabrata* was undoubtedly more resistant to a variety of cell wall stress agents, which might account for its success as a pathogen.

The cell wall also mediates the initial physical interaction between the pathogen and the host (Chaffin *et al.,* 1998) and different cell wall structures lead to different immunological responses (Lewis *et al.,* 2012). The identity of the fungal cell wall ligands that mediate the initial recognition event during host-fungus interaction remains unclear, and several conflicts in the literature about contributions of fungal cell wall components to host recognition and phagocytosis can be found (Keepler-Ross *et al.,* 2010). In this work, we demonstrated that in all described experimental conditions *C. glabrata* strains were more phagocytosed than *C. bracarensis* and *S. cerevisiae* strains. *C. glabrata* has already been reported to be more efficiently internalized by macrophages *in vitro* than *S. cerevisiae* and *C. albicans* (Keppler-Ross *et al.,* 2010; Seider *et al.,* 2014), thus, as already suggested, avoidance of phagocytosis is less likely to represent an immune evasion mechanism of *C. glabrata* (Brunke and Hube 2013). Keppler-Ross *et al.* (2010) have indicated cell wall mannan, rather than β-glucan or chitin, as a key ligand for recognition of *C. glabrata* and *C. albicans*. Moreover, these authors defended that dectin-1-activation only occurs after the initial

recognition of mannan or mannosylated proteins in murine macrophages, in contradiction to what has been stated in other studies (Herre *et al.,* 2004; Mckenzie *et al.,* 2010). Higher mannose/glucose ratio in *C. glabrata* was made responsible for the higher rates of phagocytosis by macrophages when compared with *C. albicans* (Groot *et al.,* 2008). Such an explanation would perfectly suit the differences found in *C. glabrata vs C. bracarensis* phagocytosis, but fail to explain why *S. cerevisiae*, which had a cell wall mannose/glucose ratio similar to that of *C. glabrata*, was so poorly internalized. No differences in yeast phagocytosis were found upon blocking mannan receptor with soluble mannans, though this effect could be anticipated in RAW 246.7 macrophages, which were reported as not expressing this PRR (Li *et al.,* 2010). On the opposite, pre-incubation with laminarin and with anti-Dectin-1 mAb induced a decrease in the phagocytosis rates of *C.* glabrata, *S. cerevisiae*, and *C. albicans*, but not of *C. bracarensis*. Although these results indicate that the content of β-1,3-glucan was not directly associated with the phagocytosis rates, the reason for the effect in *C. glabrata* and *S. cerevisiae* phagocytosis is somewhat ambiguous. On the one hand, RAW 246.7 macrophages express low levels of Dectin-1 (Li *et al.,* 2010), and on the other hand, *C. glabrata* and *S. cerevisiae* had the lowest β-1,3-glucan exposure. Whether laminarin and anti-Dectin-1 mAb could be inhibiting other internalization routes would be worth clarifying.

Upon interaction with pathogens, phagocytes rapidly produce ROS to destroy invading microbes (Frohner *et al.,* 2009). However, several pathogens have developed strategies to evade this microcidal mechanism. Namely, *C. albicans* and *C. glabrata,* but not *S. cerevisiae*, can actively suppress ROS production in a murine macrophage cell line (Wellington *et al.*, 2009). The recognition of components of the fungal cell wall is needed for ROS production and *Candida* viability is needed for the suppression effect. Here, it is shown that not only viable *C. glabrata* and *C. bracarensis* could suppress ROS production, but unexpectedly, *S. cerevisiae* could reduce ROS production to more than half. Heat killing of *Candida* cells, resulting in increased exposure of cell wall β-1,3-glucan, which is highly immunostimulatory, is charged for the increase in ROS production when macrophages are stimulated with HK comparatively to viable yeasts (Wellington *et al.*, 2009). This effect is most probably through phagocyte receptor Dectin-1 signalling, which besides being a phagocytic receptor, also leads to ROS and cytokine production (Ifrim, 2014; Seider *et al,* 2014; Netea *et al.,* 2015). The similar ROS production by macrophages exposed to *C. glabrata* and *C. bracarensis*, though *C. glabrata* was significantly more phagocytosed than *C. bracarensis*, could be a result of higher β-1,3-glucan accessibility in *C. bracarensis*, and

consequently higher recognition by Dectin-1 and higher ROS production. Also, a much higher β-1,3-glucan exposure in *S. cerevisiae* upon heat killing in this study, related to the one obtained in the Wellington and co-workers study, could account to the observed elevated ROS production. Alternatively, this suppressive effect on ROS production could be strain dependent, since the *S. cerevisiae* strains used here could suppress considerably the induction of this microcidal mechanism.

C. bracarensis presented a significantly higher 1,3-glucose content and higher β-1,3-glucan exposure than *C. glabrata* and *S. cerevisiae*. Several cell wall mutants with increased exposure of β-1-3-glucans exhibited increased recognition by phagocytic cells, resulting in increased phagocytosis and production of cytokines by macrophages, thus inducing a strong response by the innate immune system (Davis *et al.,* 2014). The higher content and also exposure of β-1-3-glucan in *C. bracarensis*, might have accounted for the increased levels of proinflammatory cytokines induced by this species in BMDCs and BMMs due, in part, to the immune recognition through Dectin-1, since the recognition of this cell wall component leads to the induction of proinflammatory cytokines (Netea *et al.,* 2010). Intact cells of *S. cerevisiae* and *C. albicans* have low reactivity with Dectin-1 receptor (Seider *et al.,* 2014), however, when β-glucans were more exposed, binding of Dectin-1 facilitated recognition and elicited immune cell activation and production of cytokines such as IL-23 and TNF-α. The more exposed β-1,3-glucans in the cell wall of *C. bracarensis* could thus be mediating such an effect in BMDCs and BMMs. Why dectin-1 blockage affected negatively BMDCs maturation and cytokine production by both BMDCs and BMMs but not phagocytosis by murine macrophages is somewhat puzzling. Nevertheless, these processes of the innate immune response are not necessarily positively correlated. It is already known that Dectin-1-mediated (TLR2-dependent) cytokine production in response to β-glucans can occur at the cell surface without the need for phagocytosis (Brown *et al.*, 2003). Dectin-1 mediated phagocytic clearance of fungal particles and induction of transcription of innate response genes are linked but, curiously, Dectin-1 signaling for inflammation is attenuated by phagocytosis, because blocking Dectin-1 ligand-dependent internalization on phagocytic cells leads the production of high levels of pro-inflammatory cytokines (Hernanz-Falcón *et al.*, 2009).

Given the differences in cell wall components between *C. glabrata* and *C. bracarensis*, a distinct recruitment of inflammatory leukocytes into the peritoneal cavity of mice i.p. infected with these two yeast species could be expected. Yet, no such differences could be observed regarding neutrophils, inflammatory monocytes, and macrophages recruitment. It would be interesting to

evaluate if, despite similar leukocyte recruitment, a distinct profile of cytokines and chemokines was being induced, resulting in a later disparate immune response to the two yeast species.
Altogether these results support the hypothesis that *C. bracarensis,* due to more exposed cell wall β-1-3-glucans, facilitate yeast recognition by the C-type lectin Dectin-1, which would be in agreement with the observed increased IL-23 and also TNF-α elicited in response to this species that was reduced upon Dectin-1 blockage. The higher accessibility to *C. bracarensis* β-1,3-glucans could in part be due to the higher porosity of the cell wall of this yeast species. A strong Dectin-1-mediated proinflammatory response by the host innate immune cells to *C. bracarensis* could thus contribute to a faster elimination of this pathogen. Although these results do not by themselves fully elucidate the disparate incidence of *C. glabrata vs C. bracarensis*, they nevertheless contribute to better understand the differences on the interaction of these two related species with the host.

It would be interesting to evaluate in the future if *C. bracarensis* would also lead to a marked proinflammatory response in cells of the human innate immune response. Also, if this yeast species could share *C. glabrata's* ability to interfere with the phagosome maturation and to survive within macrophages would be worth studying. Finally, a deeper characterization of the cell wall of these sibling species, namely by transmission electronic microscopy, and a comparative study of genes involved in cell wall synthesis/assembly could also contribute to clarify the disparate incidence of infection of these yeasts.

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